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FACULDADE DE CIÊNCIAS
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Brewery and olive oil industries: wastes valorisation by anaerobic digestion

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Mestrado em Microbiologia Aplicada

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2019

This Dissertation was fully performed at LNEG – National Laboratory of Energy and Geology under the direct co-supervision of Doctor Ana Eusébio and Doctor Isabel Paula Marques

Professor Margarida Barata was the internal supervisor designated in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon

Acknowledgments/agradecimentos

À Doutora Ana Eusébio por me ter dado a oportunidade de trabalhar neste projeto e também pelos conselhos que me deu ao longo destes anos, tanto na organização do trabalho como nos procedimentos. Agradeço também pelo tempo despendido e paciência que teve para comigo para me ajudar no laboratório nas situações mais complicadas, e também durante a escrita da tese.

À Doutora Isabel Paula Marques também pela oportunidade que me deu para participar no projeto, mas também pelo tempo, e paciência, que despendeu para me dar todo o apoio, conselhos e ajudas quando eu mais precisava durante o trabalho no laboratório, em termos de organização, de procedimentos e de explicar o funcionamento dos métodos e dos reatores usados, e também durante a escrita da tese.

À Natércia Santos e Graça Gomes pela assistência laboratorial e por me terem ensinado os métodos laboratoriais necessários para este trabalho. Agradeço também pelo tempo e paciência que tiveram para comigo nos primeiros meses no laboratório devido à minha falta de habilidade que tinha na altura.

À Doutora Luísa Roseiro e Doutor Luís Ramalho pelo apoio e ajuda que prestaram na avaliação dos compostos fenólicos e caracterização cromatográfica, e por me terem explicado as metodologias usadas.

Ao Doutor Francisco Gírio, coordenador da Unidade de Bioenergia por ter autorizado o estágio, e por me ter dado a oportunidade de trabalhar neste projeto.

À Professora Margarida Barata pelo apoio e orientação prestados durante este trabalho, e pelo ensino que me deu durante este mestrado que frequentei.

Ao Laboratório Nacional de Energia e Geologia (LNEG) pelas instalações e por ter fornecido o material necessário para o trabalho.

À Sociedade Central Cervejas e Bebidas (SCC), à *Valorgado*, à Companhia do azeite de Rio Maior e à SIMARSUL por terem fornecido os efluentes.

À minha família, especialmente aos meus pais e irmão, pelo todo o apoio que me deram ao longo deste trabalho, pela paciência que tiveram para comigo e por estarem sempre presentes para que eu me sentisse em casa no final de um dia de trabalho.

Aos colegas que conheci e aos amigos que fiz enquanto estive neste trabalho, à Alice, Carolina, Marta, Mariana, João, Joana, e outros tantos, pelas dicas e ajudas que me deram e pelos momentos de descontração e de convívio.

Este trabalho foi financiado através de fundos nacionais por meio da *FCT – Fundação para a Ciência e a Tecnologia, I.P.* no projeto ERANETLAC/0001/2014, GREENBIOREFINERY - Processing of brewery wastes with microalgae for producing valuable compounds.

RESUMO

A produção do azeite é uma indústria importante em Portugal, bem como em toda a região Mediterrânica. É feito a partir do fruto da azeitona (*Olea europaea*), esmagando-a e extraindo o óleo da pasta resultante através de prensagem ou centrifugação. A cerveja é uma bebida consumida mundialmente e com grande impacto económico, em Portugal inclusivamente. Na sua produção são utilizados malte e lúpulo que são fervidos em caldeiras de cobre e depois são fermentados pelas leveduras até obter o produto final. A produção suinícola é outra área comercial importante, com instalações estabelecidas no território nacional. Todas estas produções geram grandes volumes de águas residuais e, devido à sua elevada carga orgânica e toxicidade, levantam preocupações ambientais, quer das zonas aquáticas (lagos e rios, por exemplo) quer a nível do ar e solo. A fim de minimizar a potencial capacidade poluidora destes efluentes, é necessário providenciar a devida gestão tendo em conta, sempre que possível, a respetiva valorização. Este trabalho visa a valorização e tratamento de efluentes/resíduos orgânicos através da sua degradação em condições anaeróbias, como método de tratamento biológico de efluentes que utiliza microrganismos com a capacidade de degradar a matéria orgânica e compostos tóxicos na ausência de oxigénio. O processo de digestão anaeróbia foi aplicado a efluentes industriais – água ruça (OMW) e águas residuais da indústria cervejeira (BWW), obtidos da produção de azeite e de cerveja, respetivamente - por meio do conceito de complementaridade de efluentes, para melhorar o tratamento dos substratos e a produção de biogás/metano. A digestão da água ruça ("OMW") em mistura com um substrato concentrado (efluente suinícola, "PE": primeiro ensaio) foi realizada em condições mesófilas de temperatura (37°C ± 1ºC) e em condições de alimentação em descontínuo, utilizando diferentes proporções volumétricas de efluentes: 100%PE, 30%OMW+70%PE, 50%OMW+50%PE, 80%OMW+20%PE. As unidades com uma proporção baixa de OMW (30%OMW) e apenas com PE (100%PE) forneceram a maior quantidade de biogás (780 mL, 70% CH_4), enquanto que as unidades contendo 80% OMW geraram os menores volumes (120 mL, 6% CH_4), possivelmente devido à influência negativa da OMW, em elevadas quantidades, sobre as populações microbianas. Nas misturas com idênticas proporções de efluentes (50%OMW+50%PE), foi observada uma fase inicial de latência sem a produção de gás, de cerca de 25 dias, interpretada como um período de inibição do processo em que a população microbiana se foi adaptando ao longo do tempo e veio a proporcionar a evolução da produção do gás até ao volume de 327 mL (60% CH_4). Em concordância com as produções em gás, as unidades 100%PE e 30%OMW+70%PE apresentaram uma maior capacidade de remover/converter a matéria orgânica, tendo-se registado valores de 63% e 75% na Carência Química de Oxigénio (CQO), respetivamente. Nas restantes unidades, as remoções em CQO foram de 48% (50%OMW+50%PE) e 29% (80%OMW+20%PE). Quanto à atividade antioxidante (Capacidade Antioxidante Equivalente em Trolox, TEAC), verificou-se que o decréscimo originado pelo processo de digestão anaeróbia é também mais acentuado nas primeiras duas situações do que nas restantes. Alterações de 1,11 para 0,64 mmol TEAC (100%PE) e de 1,07 para 0,39 mmol TEAC (30%OMW+70%PE) foram registadas. Os resultados obtidos indicam que o efluente da indústria do azeite tem um efeito negativo sobre os microrganismos, inibindo o bom desenvolvimento do processo anaeróbio de tratamento dos efluentes, quando presente em volumes da ordem dos 50%. No entanto, verificou-se que o efluente da indústria do azeite em misturas com proporções inferiores (30%), tem efeitos benéficos e que, comparado com as unidades com apenas o efluente suinícola, revela maior capacidade de remoção da matéria orgânica, apesar de apresentar idênticas produções em biogás e metano. Na segunda experiência, o OMW foi digerido com um substrato diluído (efluente de cervejaria, "BWW"), a 37°C ± 1ºC e em condições de alimentação em descontínuo, utilizando inóculo (I) a 30% v/v. Todas as unidades testadas das diferentes misturas - 70%BWW+I, 50%BWW+20%OMW+I, 30%BWW+40%OMW+I, 10%BWW+60%OMW+I, I+H2O -

geraram baixos volumes de biogás. A produção mais elevada foi 66 mL, obtida nas unidades sem OMW (70%BWW+I). É de referir que, à semelhança da experiência anterior, na mistura com a menor proporção de OMW (50%BWW+20%OMW+I) foi identificada uma fase de inibição do processo, de cerca de 20 dias, durante a qual a população microbiana teve possibilidade de se adaptar às condições operacionais e de gerar uma produção média de biogás de 42 mL, decorridos 34 dias de ensaio. O incremento da proporção de OMW nas misturas testadas ocasionou um aumento na concentração de diversos parâmetros nos substratos a digerir (e.g. CQO, AGV, sólidos, azoto), os quais, após digestão anaeróbia, não foram sujeitos a grandes alterações, constatando-se haver uma diminuta/nula capacidade de remoção por parte do processo. O aumento da concentração em CQO e AGV, bem como o decréscimo do pH para valores na gama ácida (pH 4,8, 10%BWW+60%OMW+I), observada nas misturas com OMW, está de acordo com as baixas produções em biogás obtidas e que confirmam a ação inibidora por parte da OMW. Um aspeto relevante desta experiência diz respeito ao aparecimento de depósitos de cor avermelhada no meio de cultura. A análise microscópica mostra a existência de aglomerados da mesma cor que são identificados por espectrofotometria como corresponderem aos pigmentos bacterioclorofila a e carotenoides, típicos de bactérias púrpura não sulfúricas. Posteriormente, por análise molecular, verificou-se a presença de populações do género das *Rhodobacter*. Os resultados obtidos nesta segunda experiência, em que o OMW foi digerido com um substrato diluído (BWW), confirmam a existência de um efeito negativo por parte do OMW sobre a atividade da população microbiana, indicando que não há vantagem em usar as águas residuais de cervejaria em mistura com OMW. O reator anaeróbio híbrido foi alimentado com BWW em mistura com PE (60:40% v/v, respetivamente), tendo funcionado sob regime semi-contínuo de alimentação e com três diferentes tempos de retenção hidráulica (TRH). Operando com 5,7 dias de TRH (carga orgânica de 5,2 kg m^{-3} d^{-1}), a produção de biogás evoluiu de 0,4 para 1,2 L L^{-1} d^{-1}, com um teor em metano de 63-78%. Com a diminuição do tempo de residência para 3 dias, e o consequente aumento da carga orgânica para 10,0 kg m^{-3} d^{-1}, houve uma melhoria na produção de biogás e na sua qualidade (2,3 L L^{-1} d^{-1}, 79,5% de teor em metano). Sujeitando o reator anaeróbio híbrido a TRH de 1 dia, correspondente à máxima carga orgânica ensaiada (33,6 kg m^{-3} d^{-1}), obteve-se um novo aumento na produção de biogás, tendo-se alcançando volumes próximos de 3 L L^{-1} d^{-1} e mantido a quantidade de metano na mesma gama de valores (79,5%). No que respeita à capacidade de remoção do processo levado a cabo no híbrido anaeróbio, obtiveram-se remoções de 52% em CQO dos substratos digeridos na primeira fase da experiência (TRH=5,7d). Contudo, este comportamento não se veio a verificar nas fases seguintes devido, possivelmente, à saída de partículas/flocos do interior do híbrido em conjunto com o substrato digerido, em resultado do aumento do fluxo do alimento. O bom funcionamento do hibrido, ao longo das três condições operacionais estudadas, é suportado pela produção em biogás e metano e pela capacidade em remover/converter os AGV contidos no alimento (64 e 87-95%). A análise ao perfil do híbrido anaeróbio permitiu verificar que existe uma diminuição acentuada de AGV no troço inferior da coluna, sugerindo que estes compostos são maioritariamente degradados nesta seção da unidade. Quanto aos outros parâmetros (CQO, ST e SV), observa-se um aumento das respetivas concentrações também neste troço e que é interpretado como corresponder à existência de um manto de lamas na base da coluna. Nas restantes tomas até ao topo do híbrido, onde é recolhido o substrato tratado, verifica-se um decréscimo gradual da concentração da matéria orgânica, indicando que a degradação do substrato continua nas zonas superiores do reator. Os resultados obtidos durante a operação do híbrido anaeróbio permitem confirmar que a digestão combinada das águas residuais da produção de cerveja e da suinicultura foi realizada com sucesso mesmo quando o reator anaeróbio híbrido funcionou um tempo de residência tão baixo quanto o de 1 dia. A estrutura da comunidade microbiana foi caracterizada por Next Generation Sequencing (NGS) do gene 16S rRNA nos substratos, no inóculo e nas amostras com melhor produção de biogás/metano. Relativamente ao domínio Bacteria, Proteobacteria (54,6%) e Chloroflexi (18,4%) foram os filos dominantes detetados no inóculo. Durante os ensaios de digestão anaeróbia, as populações microbianas Pseudomonadales e

Anaerolineales, pertencentes àqueles filos, mantiveram-se predominantes em todas as amostras que foram inoculadas. Bacteroidetes (53,2%) foi o filo dominante encontrado na BWW, e Firmicutes (65,1%) em PE, mantendo-se predominantes as populações Clostridiales em todos os ensaios complementados com PE. Relativamente ao domínio Archaea, as populações dominantes pertencem aos géneros *Methanosaeta* (99,7%) e *Methanobrevibacter* (72,1%), detetadas nas amostras de inóculo e PE, respetivamente. O BWW não revelou populações de árqueas presentes nas amostras. No final do processo da digestão anaeróbia de amostra de OMW complementada com PE, detetou-se a predominância de *Methanosarcina* em todas as amostras. No segundo e terceiro ensaios, *Methanosaeta* apresentou a maior abundância relativa. A predominância de *Methanosarcina* e *Methanosaeta* é consistente com a maior produção obtida de biogás nas amostras caracterizadas.

Palavras-chave: digestão anaeróbia, biogás/metano, efluentes agropecuários e industriais, populações de bactérias e árqueas

ABSTRACT

Anaerobic digestion process was applied to the industrial effluents valorisation – olive mill wastewater (OMW) and brewery wastewater (BWW), obtained from olive oil and beer productions - by means of the effluent complementarity concept, to improve the substrates treatment and the biogas/methane production. The digestion of OMW in admixture with a concentrated substrate (piggery effluent, PE: first essay) was carried out at mesophilic conditions of temperature (37 ºC ± 1ºC) and batch conditions, using different volumetric proportions of effluents: 100%PE, 30%OMW+70%PE, 50%OMW+50%PE, 80%OMW+20%PE. The units with low proportion of OMW (30% OMW) and PE alone (100% PE) provided the highest amount of biogas (780 mL, 70% CH_4), while units containing 80% of OMW generated the lowest volumes (120 mL, 6% CH_4), possibly due to the OMW negative influence on microbial populations when present in large amounts. In the second experiment, OMW was digested with a diluted substrate (brewery wastewater, BWW), at 37 ºC ± 1ºC, under batch conditions, using inoculum (I) at 30% v/v. All tested units - 70%BWW+I, 50%BWW+20%OMW+I, 30%BWW+40%OMW+I, 10%BWW+60%OMW+I, I+H_2O - generated low volumes of biogas (less than 70 mL), confirming the negative effect of OMW on the microorganisms activity and indicating there is no advantage in using brewery wastewater in admixture with OMW. Hybrid anaerobic reactor was feed with brewery wastewater and piggery effluent mixture (60:40% v/v, respectively), under semi-continuous mode and three different hydraulic retention times (HRT). Operating at HRT of 5.7 days (loading rate of 5.2 kg m^{-3} d^{-1}), the biogas production evolved from 0.4 to 1.2 L L^{-1} d^{-1}, with a methane content of 63-78%. By decrease HRT to 3 days (loading rate of 10.0 kg m^{-3} d^{-1}), biogas production was improved and its quality as well. Biogas values of 2.3 L L^{-1} d^{-1} and methane of 79.45% were recorded. When the hybrid reactor operated with an even lower HRT (1 day), and the highest organic loading rate tested (33.6 kg m^{-3} d^{-1}), biogas production was enhanced reaching volumes close to 3 L L^{-1} d^{-1} while the methane amount was maintaining in the previous range (79.5%). The combined digestion of brewery wastewater and piggery effluent, using the hybrid anaerobic reactor, was successfully performed, even when it was working with an HRT as low as 1 day. The microbial community composition was characterized by Next Generation Sequencing of 16S rRNA gene. Proteobacteria (54.6%) and Chloroflexi (18.4%) were the dominant phyla present in the inoculum. These bacterial populations maintained their predominance in all inoculated samples during anaerobic digestion. Bacteroidetes (53.2%) was the dominant phylum found in BWW, and Firmicutes (65.1%) was the dominant phylum found in PE, maintaining its predominance in all essays complemented with PE during anaerobic digestion. Archaeal populations were only detected in inoculum and PE samples, mainly assigned to Methanosaeta (99.7%) and Methanobrevibacter genera (72.1%), respectively. At the end of anaerobic digestion of OMW complemented with PE, the genus Methanosarcina was dominant in all samples. In the second and third essays, Methanosaeta represented most of Archaea domain.

Keywords: anaerobic digestion, biogas/methane, brewery and olive mill wastewaters, piggery effluent, populations of bacteria and archaea

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1. INTRODUCTION

1.1. Olive oil industry

1.1.1. Olive oil: production and wastes

Olive oil is produced from the olive fruit (*Olea europaea*) when it's crushed and the resulting oil is extracted from the aqueous part (Kapellakis *et al.*, 2008). Since the Ancient civilizations this product has multiple uses, as food, fuel source or for cosmetics (Kapellakis *et al.*, 2008). Both the tree and the fruit still have a religious symbolism in our day. Throughout the time, the extractions techniques remained quite identical, though in the present days they have evolved to a more industrial level (Kapellakis *et al.*, 2008). In Portugal it is one of most important food industries, with 66532 tons of virgin oil produced in 2014 (FAOSTAT, 2019).

There are three olive oil's production techniques, having some processing steps similar to each other. Firstly, the olive is separated from the leaves and other residues and washed. Then follows the crushing to form a paste, to which water is added. The paste is then mixed in a process called malaxing, with the purpose of agglutinate the oil droplets to ease the aqueous phase's extraction (Kapellakis *et al.*, 2008). From this point the extraction techniques become different; the pressing technique, the most ancient, uses several stacked discs, made of synthetic fibers, and a press to crush the pulp, letting the liquid phase pass through and separating it from other phases (Dermeche *et al.*, 2013). In the end, the liquid phase is decanted to separate the oil from the aqueous phase (Olive Mill Wastewater - OMW). The remaining two techniques apply centrifugation to separate the olive oil from the aqueous phase (El Mekawy et *al.*, 2014). The three-phase technique uses two centrifugations, obtaining an olive oil with good quality. It generates two wastes (or byproducts): the olive pomace, a solid residue, from first centrifugation, and olive oil wastewater, from second centrifugation (Dermeche *et al.*, 2013). The two-phase method only uses one centrifugation and the resulting wasted flow is a dense olive wastewater. Despite being used by the majority of the largest olive oil producers' countries, the three-phase system is identified with consumption of large volumes of water and, consequently, large amounts of wastewaters to be treated (Dermeche *et al.*, 2013 and El Mekawy *et al.*, 2014). These are the reasons why two-phase system is being accepted, because of its low environment impact and low water consumption (Chowdury *et al.*, 2013 and Dermeche *et al.*, 2013).

However, there is no effective solution to get rid of these wastes mainly due to the high organic content and toxicity which make their disposal hazardous by conventional means. Several tries to give use to these byproducts such as in agriculture by spreading on olive groves, or in energy production (by incineration), but the results of the experiments are inconclusive or negative and doesn't let use them more frequently. The high content in organic compounds means this wastewater can't be discharged untreated to water courses because it'll cause hypoxia, asphyxiating fish populations, and leading to eutrophication, intensified if the waters are rich in phosphorus (Dermeche *et al.*, 2013; Kapellakis *et al.*, 2008 and McNamara *et al.*, 2008). The existence of phenolic compounds in big concentrations restricts the OMW use as a fertilizer because of their phytotoxicity, i.e. inhibits plant growth, affecting the arbuscular system and stopping seed germination (Amaral *et al.*, 2008 and McNamara *et al.*, 2008). The effluent is also toxic to microorganisms, even being capable of stopping anaerobic treatments in municipal treatment plants (Amaral *et al.*, 2008; Beccari *et al.*, 1996; Heredia and Garcia, 2005 and Paraskeva and Diamadopoulos, 2006). The dark colour, synthetized by oxidation and polymerization of tannins, creates visual pollution in waters (Kapellakis *et al.*, 2008; McNamara *et al.*, 2008 and Rahmanian *et al.*, 2014). Also, the lipids present in OMW create a film on water surface blocking solar light and oxygen from entering the water, and the oils can make the soils less capable of retain water

(Dermeche *et al.*, 2013 and Kapellakis *et al.*, 2008). And, according to El Hajjouji *et al.*, 2007, OMW is also genotoxic due to gallic acid and oleuropein presence, with lasting effects even under large dilutions (up to 10% v/v). Because of its dangers and the seasonality of oil production, the wastewater rather ends up in big open tanks, generating foul odours by fermentation of wastes and gas production, like methane and hydrogen sulphite, affecting neighbouring populations (Heredia and Garcia, 2005; Kapellakis *et al.*, 2008 and Paraskeva and Diamadopoulos, 2006).

1.1.2. Olive oil wastewater: characteristics

OMW has a reddish to black colour, given by recalcitrant compounds (lignin and other polyphenols), and cellulosic compounds (Dermeche *et al.*, 2013 and Paraskeva and Diamadopoulos, 2006). It contains water (80-95%), and is rich in organic matter, mostly sugars (fructose, mannose, glucose, saccharose, sucrose, among others), long chain and volatile fatty acids, and phenolic compounds (as mentioned earlier, they are phytotoxic) (McNamara *et al.*, 2008 and Rahmanian *et al.*, 2014). OMW has a high COD content, usually up to 200 g L^{-1}, an acidic pH (between 4 and 6) and a high solid matter (up to 20 g L^{-1}) (El Mekawy *et al.*, 2014; Eroglu *et al.*, 2006; Paraskeva and Diamadopoulos, 2006 and Tsagaraki *et al.*, 2007). Additionally, it contains minerals (potassium, sodium and calcium), fermentable proteins, resinous and serous substances, vitamins and small amounts of olive oil (Dermeche *et al.*, 2013; Kapellakis *et al.*, 2008 and Tsagaraki *et al.*, 2007). Also OMW comprises microbial inhibitors (e.g. catechol, 4-methyl-catechol and hydroxytyrosol; Tsagaraki *et al.*, 2007). The composition of this wastewater changes a lot depending mainly on the type and the maturation of olive fruit, the way of processing and handling, and the region of the olives origins. The phenolic compounds present on OMW belong to the following groups: cinnamic acid derivatives, benzoic and tyrosol compounds (Borja *et al.*, 1996). All of them have in common a hydroxyl group, an aromatic ring and a functional chain. They are found in olive fruit under glucoside, tannins, anthocyanins and lignin forms. Their role is protecting the fruit from oxidation, microorganisms and UV light, while also give a lighter colour (Dermeche *et al.*, 2013; El Mekawy *et al.*, 2014; Kapellakis *et al.*, 2008 and Morillo *et al.*, 2009). These characteristics described here mean that this effluent must be treated before being disposed safely.

1.2. Brewery industry

1.2.1. Brewery: production and wastewater

Beer is one of the most ancient and consumed beverages worldwide, being one of the most valuable in food industry, including Portugal, where 729000 tons of beer were produced in 2014 (FAOSTAT, 2019). Its production usually evolves malt, hops, yeasts of *Saccharomyces* genus, and some sugars or starch to add some flavour to the beer (Brito *et al.*, 2007). The production begins when malted barley grains are mixed with water and crushed; the resulting sugars are retrieved along with water. This mixture is then boiled in copper kettles and hop is added for the liquid to get the characteristic bitterness. Finally, the mixture is fermented by yeasts and stored in barrels or in bottles.

The production of this beverage always evolves big volumes of water, and adding the water spent on washing bottles, tanks and machines (3 – 10 L for every litre of beer), the result is high volumes of wastewater discharged annually, which create pollution concerns such as eutrophication of water bodies and oxygen depletion (Simate *et al.*, 2011).

1.2.2. Brewery wastewater: characteristics

Brewery effluent contains a great amount of organic compounds: volatile fatty acids, sugars, starches, ethanol, among others. The solid compounds consist of spent grains, waste yeast and hot trub (Brito *et al.*, 2007 and Simate *et al.*, 2011). The COD of the wastewater is typically 2 – 6 g L^{-1}, depending of its

origins (Brito *et al.*, 2007). The effluent's pH can have big variations, between pH 2 – 12, depending on chemicals used for cleaning (acids, caustic soda and chlorine, for example) and disinfection of microorganisms, except yeasts (Brito *et al.*, 2007 and Simate *et al.*, 2011). The effluent's temperature also varies between 18ºC and 38ºC (Brito *et al*., 2007). Moreover, nitrogen and phosphorus' levels on the effluent are dependent of the quantity of spent yeast used and the way it was handled. In general, the brewery wastewater's composition varies largely in many parameters (Simate *et al.*, 2011).
Brewery effluent's composition makes this flow dangerous to be discharged without any management step. To avoid and/or reduce the problem it is necessary to provide wastewater treatment.

1.3. Pig industry

1.3.1. Pig industry and piggery effluent

Pig industry is one of leading animal and food economies worldwide as well in Portugal, where about 2615000 heads in 2017 (FAOSTAT, 2019) accounted for 1.2% of European production. With this large and intensive production, it's clear that big amounts of manure will be produced and mostly will go to the sewage. Another form of disposing, used by the farmers, is by landfilling it in the crops as a fertilizer. However, the manure poses many dangers as it is a very strong pollutant for water courses and soil. It can cause eutrophication in watercourses and water bodies due to nitrogen (Bernet and Béline, 2009 and Prapaspongsa *et al.*, 2010). If land spreaded without precautions it will contaminate the soil, and underground water bodies due to excess in phosphorous and nitrogen compounds (Bernet and Béline, 2009 and Prapaspongsa *et al.*, 2010). Piggery effluent is also responsible for gas emissions, notably greenhouse gases and nitrogen-based gas (ammonia and nitrous oxide), and foul odours (Bernet and Béline, 2009 and Prapaspongsa *et al.*, 2010). So, it is always necessary to provide treatment process the manure in order to safely dispose the pig industry effluents.

1.3.2. Piggery effluent: characteristics

The piggery effluent (PE) is an animal residue and it consists of pig excretions. Usually, the solid fraction is composed by animal excretions and food leftovers, and the liquid fraction is composed by urine and water from washing and disinfections. Usually it is rich in organic matter, solid residues (usually above 40 g L^{-1}) and nitrogen (mostly ammonia) (Bernet and Béline, 2009; Boopathy, 1998 and Hwang *et al.*, 2010). Also it contains high amounts of phosphorus and potassium, and other compounds that create foul odours (e.g. volatile fatty acids and phenolic compounds) as well. This means that this waste has a high COD and a pH around 7.5 (Beaudet *et al.*, 1990; Bernet and Béline, 2009; Boopathy, 1998; Hatfield *et al.*, 1998 and Zhu, 2000). Additionally, proteins, lipids and cellulosic residues, are also present (Boopathy, 1998). It also contains many microorganisms that are potentially pathogenic for humans, such as bacteria and virus, and also protozoans (Beaudet *et al.*, 1990; Bernet and Béline, 2009; Pagilla *et al.*, 2000 and Zhu, 2000).

1.4. Effluent treatment

There are many methods to provide the treatment of the effluents, and they can be physical, physio-chemical and biological.

1.4.1. Physio-chemical treatments

Physical methods are used to treat wastes. Some of them are filtration, centrifugation, incineration and sedimentation (Paraskeva and Diamadopoulos, 2006 and Simate *et al.*, 2011). Membrane filtration is another physical method in which separates bigger compounds from residual emulsion through porous

membranes (Simate *et al.*, 2011). However, they are scarcely used without being associated with other methods because they can remove solid matters and suspended particles but they are rather inefficient to remove dissolved pollutants from the effluents (Paraskeva and Diamadopoulos, 2006 and Simate *et al.*, 2011). In physio-chemical methods, both physical and chemical processes are involved and generally used to treat the wastes. Usually, the chemical treatments take priority in detriment of physical only. The pH can be adjusted given the initial value of the effluent. For example, carbon dioxide can be used to neutralize caustic effluents or making them more acidic if it's going to be treated in a bioreactor (Simate *et al.*, 2011). Coagulation and flocculation consists in aggregating and sedimenting organic compounds and/or colloidal substances by using coagulants. Fenton reaction (uses oxidation with free radicals), the electrochemical oxidation (using electricity as a way to eliminate the toxic compounds from the effluent), the electrocoagulation (an electrode produces a coagulant), the adsorption and ionic exchange are other examples of the physio-chemical methods (Kapellakis *et al.*, 2008 and Paraskeva and Diamadopoulos, 2006).

1.4.2. Biological treatment

Biological methods involve microorganisms to degrade the effluents' organic compounds. Compared to other processes, it has the ability to remove more organic loads at lower costs, in exchange for higher energy input (Paraskeva and Diamadopoulos, 2006 and Simate *et al.*, 2011). These processes are divided in aerobic and anaerobic digestion.

Aerobic digestion uses microorganisms, mainly bacteria and fungi, which use oxygen to degrade the wastes materials to inorganic end-products (Simate *et al.*, 2011). Activated sludge in aerated tanks and biofilms are some examples of aerobic digestion (Simate *et al.*, 2011). However, an adaptation period is always necessary for the aerobic microorganisms to be able to remove or convert organic compounds (Paraskeva and Diamadopoulos, 2006). Anaerobic digestion is having an increasing adhesion in treating effluents because, in general, it can eliminate more effluent organic load in relation to aerobic digestion, allowing to recover chemical energy in form of methane, and with the possibility to combine with other pre-treatment processes (Kapellakis *et al.*, 2008). The biogas produced from this process is mostly used as a fuel, for electric and thermic energy, the latter frequently directed for heating the reactor itself (Holm-Nielsen *et al.*, 2009). Additionally, the digested flow can be reused as fertilizer in agricultural production (Holm-Nielsen *et al.*, 2009). Anaerobic digestion uses anaerobic bacteria and archaea, which have low rate of growth and low energetic needs compared to aerobic microorganisms (Paraskeva and Diamadopoulos, 2006), and can be applied to a wide variety of residues. The most used are farming/cattle manures (for example swine and bovine) and municipal wastewaters (Holm-Nielsen *et al.*, 2009 and Mao *et al.*, 2015). Anaerobic digestion can occur at thermophilic (40 – 70ºC) or mesophilic (20 – 40ºC) conditions of temperature. Thermophilic anaerobic digestion has been accepted because it can provide greater biogas production rate and due to the ability of supporting high loads than mesophilic conditions. However, it has a tendency to acidify and needs to spend more energy to maintain the temperature (Mao *et al.*, 2015). Mesophilic digestion also has been widely used due to its better stability, and greater microorganisms' diversity, although it is less efficient in biodegrading effluents' compounds and provide a lower methane production (Mao *et al.*, 2015). Others drawbacks of the mesophilic digestion lies in its inability to inactivate the all effluent pathogens because of its low working temperature, (50ºC at least is necessary, only achievable in thermophilic digestions) and the need with longer retention times (Pagilla *et al.*, 2000). It has been made experiments at 30ºC for mesophilic temperatures, but it was verified that the optimal temperature, for better methane yield, is close to 40ºC (Sakar *et al.*, 2009).

Anaerobic digestion can be described in three phases, all happening in sealed tanks and without oxygen, as shown in Figure. 1.1.: the first phase, the hydrolysis or liquefaction, the bacteria, able to ferment and release hydrolytic enzymes, degrade complex compounds (like polysaccharides, polyphenols, among others) into soluble monomers, mainly monossacharides, long chain acids, alcohols and amino acids. The second phase, the acidogenesis, and acetogenesis, acetogenic bacteria convert the latter compounds into alcohols and organic acids (including acetate), and H_2. The last phase, considered the most important, methanogenic archaea and bacteria degrade acids and acetates into methane and carbon dioxide (Ahring, 2003; Hwang *et al.*, 2010; Kapellakis *et al.*, 2008; Kim *et al.*, 2010; Kim *et al.*, 2013 and Paraskeva and Diamadopoulos, 2006). Between these three groups there is a delicate balance to be respected in order to get the maximum yield in producing biogas from the organic load of residues used as substrates. The second group relies on the ability of the third group of microorganisms in removing hydrogen from the medium; so, the less hydrogen concentration bigger is the degradation of volatile fatty acids by the second group (Ahring, 2003). However, the bacteria (and archaea) used for this process, especially the methanogens, are sensitive to variations inside the reactor. One example is the pH, their working range is between pH 6.5 – 7.8 (Sakar *et al.*, 2009). So it is important to maintain the conditions stable due to sensitivity of microorganisms; if a change occurs, the process would become unstable, resulting in low biogas yields (Mao *et al.*, 2015).

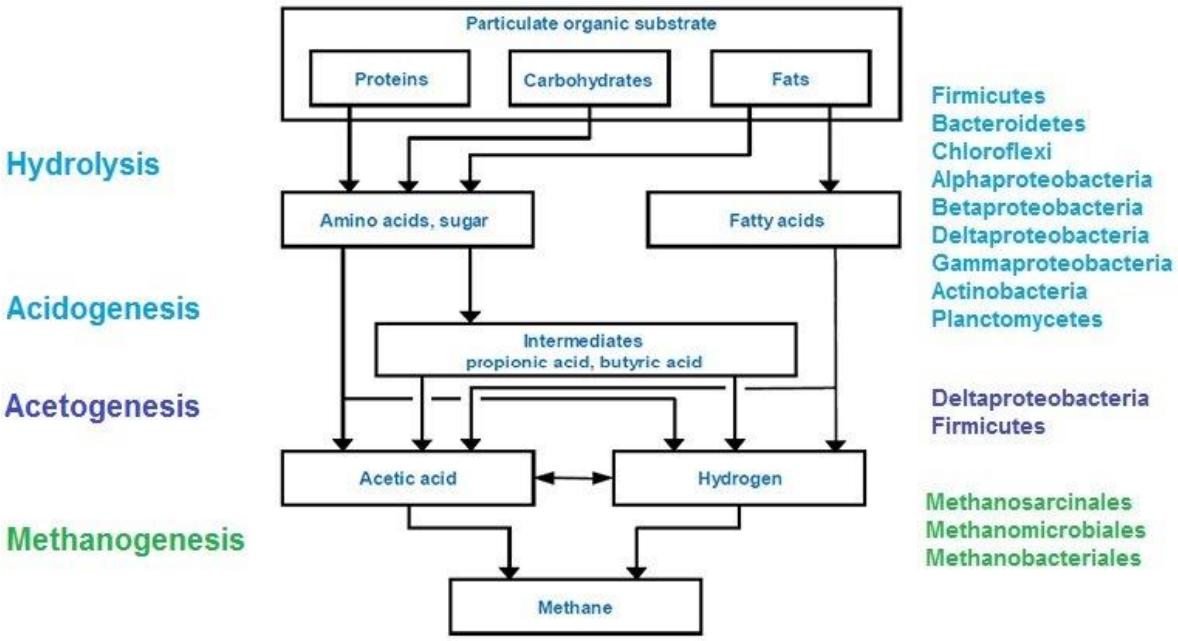

Fig. 1.1.: Anaerobic digestion flowchart (Prakash *et al.*, 2015 and Cai *et al*., 2016, adapted)

1.5. Scope and objectives of the study

The principal objective of this study is to valorise energetically organic effluents - olive oil and beer industries - through the anaerobic digestion to the biogas/methane production, by means of the effluent complementarity concept. The concept of effluent complementarity has been studied and applied in anaerobic digestions of unbalanced and/or concentrated substrates, with the intention of adding certain deficit components into an effluent and/or diluting it, using another effluent/residue. In Marques, 2001 and Sampaio *et al.*, 2011, piggery effluent was used to complement and dilute OMW. Further advantages include lowering costs, avoid substrate chemical adjustments and/or pre-treatments and improve biogas and methane production.

The present work aims to reduce the toxicity of OMW and provide nutrients, by using a concentrated and diluted substrate (piggery effluent and brewery wastewater, respectively), under batch conditions, as shown in Table 1.1. On the other hand, it was intended to test the brewery effluent as a dilution element of a concentrated substrate (piggery effluent), under semi-continuous feed conditions, according to Table 1.2.

Table 1.1.: Anaerobic digestion in batch conditions

Experiments	Effluents/inoculum	Days	Essays/mixtures	Abbreviations
I	-Olive mill wastewater (OMW) -Piggery effluent (PE)	0-73	- 100% PE - 30% OMW + 70% PE - 50% OMW + 50% PE - 80% OMW + 20% PE	-100%PE -30%O+70%PE -50%O+50%PE -80%O+20%PE
II	-Olive mill wastewater (OMW) -Brewery wastewater (BWW) -Inoculum (I)	0-34	- 30% I + H2O - 70% BWW + 30% Inoculum - 50% BWW + 20% OMW + 30% Inoculum - 30% BWW + 40% OMW + 30% Inoculum - 10% BWW + 60% OMW + 30% Inoculum	-I+H_2O -70%BW+I -50%BW+20%O+I -30%BW+40%O+I -10%BW+60%O+I

Table 1.2.: Anaerobic digestion in semi-continuous conditions for the hybrid anaerobic reactor

Experiment	Effluents	Conditions of treatment	Substrate	Days	HRT
III	-Brewery wastewater -Piggery effluent	Anaerobic digestion in semi-continuous conditions, different HRT (5.7, 3.0 and 1.0 days)	40% PE + 60% BWW	0-19 20-37 41-47	5.7 3.0 1.0

HRT = hydraulic retention time

2. MATERIAL AND METHODS

2.1. Substrates and inoculum sampling

The olive mill wastewater (OMW) was collected from an olive mill located in Rio Maior, Portugal, which work with three-phase continuous extraction process. The brewery wastewater (BWW), which was submitted to a primary treatment stage, was provided by Sociedade Central de Cervejas e Bebidas (SCC) (Vialonga, Portugal). Piggery effluent (PE) was collect at Valorgado (Salvaterra de Magos, Portugal). Valorgado has 9000 animals and the effluent produced by the farm swine facilities is estimated at 900 – 1700 m^3d^{-1}, being piped and separated into two fractions, one solid and another liquid. Experiment substrate was the liquid fraction after a removal operation of main solids by a solid-liquid separator. It was conducted by plumbing to the first pond. Experiment samples were collected at the top of this pond feed tube, making his PE an atypically concentrated piggery effluent. Biologic solids, from an anaerobic digester of a wastewater treatment plant (SIMARSUL, Quinta do Conde, Portugal), were used as inoculum (I).

2.2. Anaerobic digestion experimental set-up

In the first essay, PE and OMW were used as the substrates. The digestion was performed in triplicates under batch conditions, using glass flasks with 165 mL total volume, leaving 125 mL for headspace. Different mixtures by increasing the OMW from amounts of 30% to 80% and decreasing the PE from 70% to 20%. The essay mixtures were reported as: 30% OMW + 70% PE, 50% OMW + 50% PE, 80% OMW + 20% PE, and 100% PE for control (Table 1.1.).

In the second essay, OMW and BWW were used as the substrates, and mixtures were inoculated at 30%. The digestion was performed in triplicate under batch conditions, using glass flasks with 71.5 mL total volume, leaving 31.5 mL for headspace. Different mixtures were obtained by increasing OMW from

amounts of 20% to 60%, and decreasing BWW from 70% to 10%. The following mixtures were as reported: 70% BWW + 30% I, 50% BWW + 20% OMW + 30% I, 30% BWW + 40% OMW + 30% I, 10% BWW + 60% OMW + 30% I and 30% I + 70% H_2O for control (Table 1.1.).

All mixtures and flasks from both essays were deaerated with nitrogen gas and sealed to ensure anaerobic conditions. The flasks were incubated at constant temperature of 37 ± 1ºC. The biogas production was monitored daily with a pressure transducer, expressed to standard conditions of temperature and pressure (STP: 0ºC, 1 bar).

The third essay was sub divided into three phases, related to the time the substrate was maintained inside the reactor, i.e., the hydraulic retention time (HRT), as shown in Table 1.2. The first phase corresponds to HRT of 5.7 days, the second phase was 3.0 days, and the final phase was 1.0 day. A hybrid anaerobic reactor, designed and tested in LNEG, was used in this essay (Figure 2.1.). It has 1.7 L of useful volume, with a total of 2 L, and it's equipped with a packed bed on the upper 1/3 of the reactor height, which was selected from previous studies (Marques, 2001). Also, it had no solid/liquid/gas separator device nor substrate recycler installed. For the substrate a mixture of BWW and PE was made (40% PE and 60% BWW, v/v) to feed the reactor. The reactor was fed with substrate in up-flow mode through peristaltic pump, and digested in fed-batch conditions. The reactor's temperature was maintained at 37ºC, by using a water jacket, throughout the experiment. Before the experiment, PE was only used in the start-up phase of the reactor (data not shown). To measure the biogas production a wet gas meter was used, expressed to standard conditions of temperature and pressure (STP: 0ºC, 1 bar). Additionally, some samples were collected during the essay from the inside of the reactor to determine its profile.

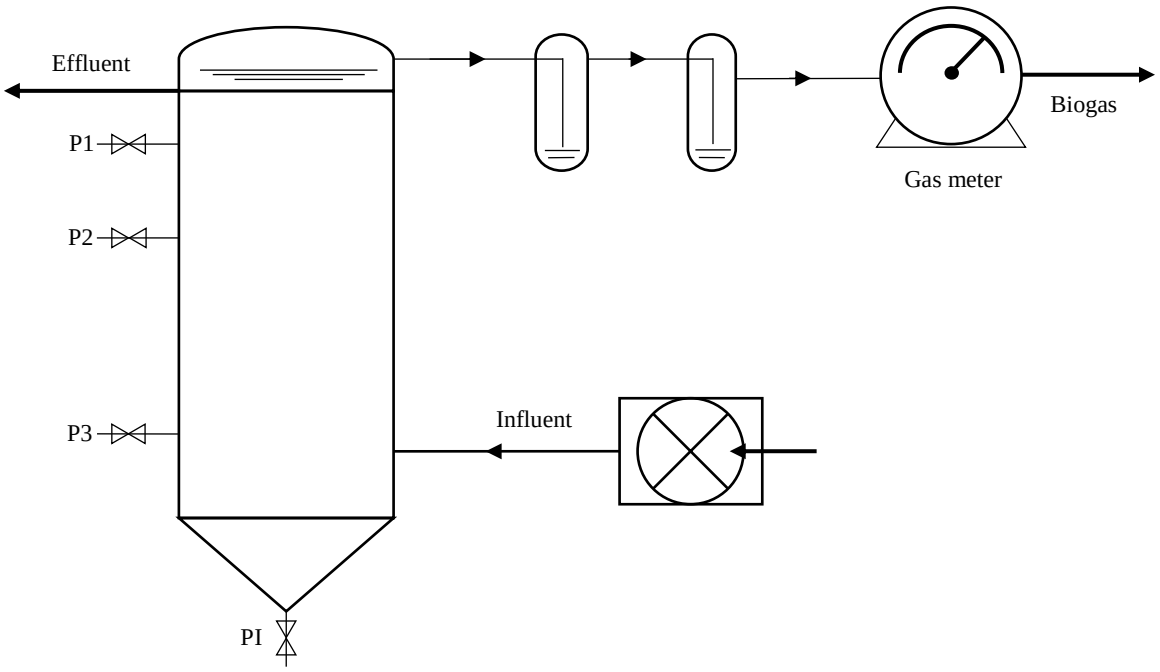

Fig. 2.1.: Anaerobic hybrid reactor schematic used for the third experiment

2.3. Analytical and chromatograph methods

2.3.1. pH

The pH of the effluents and the mixtures for anaerobic essays was measured with SenTix 41-3 (WTW) pH electrode, according to Standard Methods (APHA, 2012), before and after each experiment.

2.3.2. Total and volatile solids

Total solids (TS) is the term applied to the solid residue left in the vessel after drying and evaporation of a sample in an oven, at a defined temperature. Total solids includes ‘‘total suspended solids,’’ the portion of total solids retained by a filter, and ‘‘total dissolved solids’’, the portion that passes through the filter.

Total Solids were assayed according to Standard Methods (APHA, 2012). For each experiment, 10 or 20 mL samples were weighed in crucibles, in duplicates, and dried at 103-105ºC overnight to evaporate all water. The residue was cooled, weighed, and total solids were determined. To measure Volatile Solids (VS), an ignition at 550ºC for 1 hour was made in a muffle furnace, and weighed after cooling down (APHA, 2012). The total and volatile solids were determined by comparing the mass of the sample before and after each drying step.

2.3.3. Chemical oxygen demand

Chemical oxygen demand (COD) is defined as the quantity of oxygen, which is required to oxidize the organic matter present in a sample under controlled conditions (temperature, time and oxidizing agent). For the chemical oxygen demand, the open reflux method, with potassium dichromate, was used, according to Standard Methods (APHA, 2012), as follows:

20 mL of sample, in duplicates, were diluted 1:50 (1:100, when samples had high organic load). In each COD tube, containing diluted sample, 0.5 g of $HgSO_4$ and 5 mL of $AgSO_4$ 6.6 g L^{-1} in H_2SO_4 were added. Then 10 mL of $K_2Cr_2O_7$ (0.25 N) and 25 mL of the same acid solution were added. The tubes were refluxed at 150ºC for 2 hours, cooling down, and distilled water was added until final volume of 400 mL plus 4 drops of ferroin. The color change is sharp, going from blue-green to reddish-brown. Then titration proceeded with a ferrous ammonium sulphate solution (0.25 N). In order to determine the exact normality of ferrous ammonium sulphate solution, a titrand was made with 25 mL of sulfuric acid solution, 10 mL $K_2Cr_2O_7$ (0.25 N) and 400 mL of distilled water and some ferroin’s drops. The COD of the sample is given as follows (Equation 2.1.):

$$COD\ (mg/L) = [(V_A - V_B) \times T \times 8000 \times DF]/V$$

Equation 2.1.: COD equation

where, V_A = Volume, in mL, of ferrous ammonium sulfate solution required for titration of the blank; V_B = Volume, in mL, of ferrous ammonium sulfate solution required for titration of sample; T = Normality of ferrous ammonium sulfate solution; DF = Dilution factor, if appropriate; V = Volume, in mL, of sample used for the test.

2.3.4. Total phenolic content and electrophoretic profile

Total phenolic content was determined by a colorimetric method (Singleton and Rossi, 1965), using caffeic acid as standard for calibration curve. The samples were centrifuged at 13000 rpm for 10 minutes, then they were filtered in a GF/C fibre glass (1.25 μm pores) filters (Whatman), and diluted (1:20, or 1:50 for higher phenolic concentration). A main solution of caffeic acid was prepared (500 mg L^{-1}), diluted 1:5; and different concentrations (0 – 100 μg mL^{-1}) of the same solution were prepared for the calibration curve. For each sample tube and calibration curve tube (1 mL) the next steps were as follows: addition of 5 mL of Folïn-Ciocalteau reagent (diluted 1:10), vortex and pause for 3 minutes; addition of 4 mL of Na_2CO_3 (7.5% m/v), vortex and pause for 2 hours. Concentration of total phenols was determined using the calibration curve of caffeic acid, at 765 nm wavelength.

Phenolic profiles were obtained by capillary zone electrophoresis (CZE), using an Agilent system equipped with DAD. CZE separation was performed using a fused-silica uncoated capillary with extended light path (i.d. 50 µm, 62/56-cm length). The samples were injected at 50 mbar for 6 s under a voltage of 30 kV. The temperature was maintained at 30°C. The electrolyte (15 mM borate in 10% MeOH) was adjusted to pH 9.1. The capillary was pre-conditioned by flushing with 0.1 M NaOH for 3 min and subsequently running buffer for 3 min. Compounds were detected at 200 and 280 nm and identified by comparison of their UV spectra and migration times to authentic standards. The resulting graphics are found in Appendices section.

2.3.5. Total nitrogen (kjeldahl)

To measure total nitrogen in effluents and samples this analysis was made according to Standard Methods (APHA, 2012). 5 mL of sample, in duplicates, were put in Kjeldahl tubes, in which 50 mL digestion reagent (134 g K_2SO_4, 200 mL H_2SO_4 and 2 g HgO, for 1 L solution) was added. The digestion was made in vacuum and in constant heating until the samples acquired transparent colour. After that 100 mL of distilled water without ammonia and some drops of phenolphthalein were added. To distil the nitrogen from the samples an automatic distiller unit (Büchi Distillation Unit K-350) was used with 50 mL of boric acid indicator mix solution (20 g of H_3BO_3, 10 mL of indicator mix of methyl red and blue methylene, for 1 L) to retain nitrogen. Also 50 mL of thiosulphate hydroxide was added to samples. The Erlenmeyer with the boric acid solution was then titrated using H_2SO_4 0.02 N (or 0.1 N if the samples had high concentration of nitrogen) standard solution as titrant. The total nitrogen of the sample is given as follows (Equation 2.2.):

$$\text{Organic N (mg/L)} = [(V_D - V_E) \times 280]/V$$

Equation 2.2.: Kjeldahl nitrogen equation

where, V_D = Volume, in mL, of H_2SO_4 solution required for titration of sample; V_E = Volume, in mL, of H_2SO_4 solution required for titration of the blank; V = Volume, in mL, of sample used for the test.

2.3.6. Ammonia nitrogen

The ammonia nitrogen methods was described in Standard Methods (APHA, 2012). For each sample (10 mL), in duplicates, 140 mL of distilled water without ammonia and 25 mL borate buffer (88 mL NaOH 0.1N + 500 mL $Na_2B_4O_7.10H_2O$ 0.025 M, for 1 L) were added. The distillation and titration were made in similar fashion as total nitrogen (cf. Total nitrogen), using only the boric acid solution.

2.3.7. Volatile fatty acids

To determine the presence of volatile fatty acids (VFA) 1 mL of each sample was taken and centrifuged at 13000 rpm for 15 minutes, decanted and centrifuged again, collecting the final supernatant. Then some drops of orthophosphoric acid (85% concentrated), diluted with water 1:1 were added to samples until reach the pH 2. Analyses of VFAs were performed using a gaseous chromatograph Hewlett-Packard 5890 GC-FID and a Shimadzu C-R5A register/integrator. An internal standard solution (100 µL pivalic acid 0.1% p/p) was added to the sample (400 µL), and 1 µL from the solution was injected in chromatograph. For each standard solution, 200 mg L^{-1} of each acid (acetic, propionic, isobutyric and butyric, with 0.1% p/p concentration) was used. Total VFA concentrations were expressed as acetic acid.

2.3.8. Biogas composition

To monitor the biogas composition in terms of methane and carbon dioxide, 0.2 mL gas samples were taken from the digester headspace and analysed weekly by gas chromatographic techniques (Varian 430-

GC, TDC; HP-5890, FID), according to ASTM Standard Method (D1946–90, 2000). Gas chromatograph was equipped with a thermal conductivity detector and a Porapack S column of 1/8"x 3 m. Column, injector and detector temperatures were 50, 60 and 100ºC, respectively. Nitrogen was utilized as the carrier gas (20 mL mn^{-1}).

2.3.9. Antioxidant activity

To measure the antioxidant capacity, a radical scavenging activity against stable DPPH radical (2,2-diphenyl-2-picrylhydrazyl hydrate) method was used (Brand-Williams *et al.*, 1995). Initially, 20 mL of mixtures and effluents were freeze-dried and weighed (approximately 0.5 g for all samples, but sometimes less due to low volume available or low amount of contents). Then they were dissolved in distilled water and/or methanol (at least 5 mL of volume). If it still had sediments, only the supernatant was used. 50 µL of sample were added in a tube, in triplicates, and added 1950 µL DPPH 0.06 mM solution (prepared daily). A calibration curve was made using a Trolox 1.5 mM solution as standard antioxidant (dissolved in ethanol), made in triplicate (each with 50 µL of solution) and adding 1950 µL of the same DPPH solution. Then the tubes were kept in dark for 30 minutes, and lastly, the absorption was measured spectrophotometrically at 515 nm wavelength, in a microplate reader Multiskan GO (Thermo Scientific). An increase of DPPH radical scavenging activity was detected by a decrease of DPPH solution absorbance. The radical activity was calculated by the following equation (Equation 2.3.):

$$\%\text{DPPH inhibition} = [(Abs_b - Abs_s)/ Abs_b] \times 100$$

Equation 2.3.: Antioxidant activity equation

where, Abs_b is the absorption of blank (t = 0 min) and Abs_s is the absorption of tested solution or sample (t = 30 min). The antioxidant activity was expressed in mmol TEAC (Trolox Equivalent Antioxidant Capacity).

2.3.10. Microscopy

A sample of 70%BWW+I mixture was collected after the end of the experiment, and the observation was made under optical microscopy (Olympus BX51), with a 40x ocular making a 400x total amplification.

2.3.11. Analysis of pigments

To assess the presence of pigments an aliquot of culture medium, from the 70%BWW+I mixture after the experiment, was collected and diluted in water. After that, the absorption spectrum of intact cells was measured within a range of 380 – 900 nm (Shimadzu UV – 2401PC).

2.4. Molecular analysis

2.4.1. DNA extraction

To evaluate the biodiversity present in effluents and mixtures DNA extraction was made based in method described by Zhou *et al.*, 1996, and adapted for this samples (Eusébio *et al.*, 2011).

The process begun with centrifuging 50 mL of samples (10000 rpm, 20 minutes), in Oakridge tubes. The pellet was ressuspended in 10 mL Extraction Buffer (100 mM Tris.HCl [pH 8.0], 100 mM EDTA Na [pH 8.0], 100 mM $NaPO_4$ [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 µL Proteinase K (10 mg mL^{-1}), and incubated at 37ºC and shaked at 225 rpm, for 30 minutes. Then SDS 20% was added (1.5 mL/10 mL sample) and incubated in water-bath at 65ºC for 2 hours. Then the sample was centrifuged 10000 rpm for 10 minutes and the supernatant distributed into eppendorfs (600 µL each). The pellet was

extracted again by repeating the following two times: 4.5 mL of Extraction Buffer and 0.5 mL SDS 20%, vortex 10 seconds, incubated in 65ºC water-bath for 10 minutes and centrifuged 10000 rpm for 10 minutes. The supernatant from these extraction cycles was distributed through eppendorfs, an equal volume of chloroform-isoamyl alcohol (24:1) was added, and centrifuged at 13000 rpm for 10 minutes; the aqueous phase was collected and this process was repeated once more. The aqueous phase was precipitated with 0.6 volume of isopropanol and 0.1 volume of 2.5 M $C_2H_3NaO_2$ solution and was kept at -20ºC for 1h. The pellet of crude nucleic acids was recovered centrifuging at 13000 rpm for 20 minutes, and added cold ethanol 70%, left overnight at -20ºC, then centrifuged 13000 rpm, for 20 minutes, ressuspended in TE solution (10 mM Tris.HCl [pH 7.5], 1 mM EDTA), and stored at -20ºC.
There were made optimizations of the initial method in order to optimize the yield and quality of DNA extracted. The purity was controlled through absorbance readings at 230, 260 and 280 nm, also to determine 260/280 and 260/230 ratios for protein and polysaccharide/polyphenols contaminations, respectively. If the ratio for 260/280 is superior than 1.8 and 260/230 is superior than 2.0, then the extracted DNA is considered pure. Otherwise, if it's below it is contaminated by either proteins or humic acids, or both. Also, the reading at 260 nm gives the estimate amount of DNA present in the obtained extract (A260 = 1.0 means that there are 50 µg of DNA).
In order to avoid contamination, decrease smearing and increase integrity of genomic DNA after extraction, several trials were essayed with samples with more phenolic content and organic load, as follows:

- A) 2% CTAB on Extraction Buffer solution to eliminate humic acids from the samples (e.g. samples from piggery effluent);
- B) 2% CTAB, 100 µL lysozyme (25 mg mL^{-1}) to eliminate the protein contaminants and 5 µL RNAse A (10 mg mL^{-1}) after isopropanol addition (e.g. samples from piggery effluent);
- C) 2% CTAB, 5 µL RNAse (10 mg mL^{-1}) added between chloroform-isoamyl alcohol steps, and without the extract being overnight at -20ºC after ethanol addition (e.g. samples from OMW);
- D) 2% CTAB, 75 µL RNAse A added after chloroform-isoamyl alcohol steps and the extract was left 1h at -20ºC after ethanol addition (e.g. samples from all effluents);
- E) Similar to the latter, but with 3% CTAB to eliminate the humic acids/polyphenols from the extracts that came from samples with the highest amount of them.

2.4.2. Next generation sequencing

The sequencing of the extracted DNA was carried out on Stab Vida facilities (Lisbon, Portugal) using MiSeq (Next Generation Sequencing, NGS), as followed:
the extracted DNA was pooled, quantified and checked for purity using Qubit™ (Thermo Fisher Scientific) prior to storage at −20°C. For NGS, V3 and V4 regions of bacterial and archaeal 16S rRNA gene were amplified with universal primers 515F - 806R. Library construction was performed using the Illumina 16S Metagenomic Sequencing Library preparation protocol. The generated DNA fragments (DNA libraries) were sequenced with MiSeq Reagent Kit v3 in the lllumina MiSeq platform, using 300 bp paired-end sequencing reads.
The bioinformatics analysis of the generated raw sequence data was carried out using the Quantitative Insights Into Microbial Ecology (QIIME2, version 2018.11) (Caporaso *et al.*, 2010). The reads were denoised using the *Divisive Amplicon Denoising Algorithm 2* (DADA2*)* plugin (Callahan *et al.*, 2016), where the following processes were applied: Trimming and truncating low quality regions; dereplicating the reads; filtering chimeras. After denoising, the reads were organized in features, which are operational taxonomic units (OTUs) and a feature Table was generated using the plugin *feature-Table* (https://github.com/qiime2/q2-feature-Table), with each feature being represented by exactly one sequence. After applying the plugins *Alignment* (Katoh and Standley, 2013), *Phylogeny* (Price *et al.*,

2010) and *Diversity* (https://github.com/qiime2/q2-diversity), a pre-trained sk-learn classifier (Pedregosa *et al.*, 2011) based on the SILVA (Glöckner *et al.*, 2017) (release 132 QIIME) with a clustering threshold of 97% similarity was applied to generate taxonomy Tables. Taxonomic classification was achieved by using plugins *Feature-classifier* (https://github.com/qiime2/q2- feature-classifier) and *Taxa* (https://github.com/ qiime2/q2-taxa) where only OTUs containing at least 10 sequence reads were considered as significant.

3. RESULTS AND DISCUSSION

3.1. Effluents

PE and OMW are substrates with very high organic compounds concentrations (93 and 106 g L^{-1} COD, respectively), as shown in Table 3.1., indicating a great potential for biogas/methane production. On the other hand, these effluent present complementary characteristics in terms of composition that can be used advantageously to balance the conditions of anaerobic digestion process. Effectively, the inhibitory capacity of OMW, due to the total phenolic concentration (about 3 g L^{-1}, and containing tyrosol,

Table 3.1.: Characteristics of the effluents and inoculum

Effluents	COD (gL^{-1})	Total Solids (gL^{-1})	Volatile Solids (gL^{-1})	Total nitrogen (mgL^{-1})	Ammonium nitrogen (mgL^{-1})
PE	93.22 ± 5.01	47.4 ± 0.8	31.9 ± 0.6	4900.0 ± 277.2	3206.0 ± 19.8
OMW	105.79 ± 1.00	31.8 ± 0.0	26.1 ± 0.2	212.8 ± 15.8	1.4 ± 1.6
BWW	7.37 ± 0.00	3.6 ± 0.1	1.3 ± 0.1	25.2 ± 4.0	7.0 ± 2.0
I	17.55 ± 0.38	12.5 ± 0.1	9.6 ± 0.0	784.0 ± 79.2	351.4 ± 2.0

PE = piggery effluent; OMW = olive mill wastewater; BWW = brewery wastewater; I = inoculum; COD = chemical oxygen demand

hydroxytyrosol and its derivatives, shown in Table 3.2. and Appendices figure 1.b.), associated with an acid pH, can be minimized by addition of PE. PE is also characterized by a good antioxidant activity, with more than 80% inhibition of the radical (Table 3.2.). Furthermore, the OMW nitrogen lack (determined through total and ammonia nitrogen) may also be compensated by the presence of the high nitrogen content of PE (4.9 g L^{-1} in total, 3.2 g L^{-1} in ammonium; Table 3.1.).

BWW is a very diluted effluent and, comparatively, it holds the lowest concentration of organic materials (7 g L^{-1} COD, 4 g L^{-1} TS and 1 g L^{-1} VS), being characterized by an acid pH, low nitrogen content and a lack of phenolic contents (Tables 3.1., 3.2. and 3.3.). These features are justified since the effluent was previously submitted to a primary treatment, before to be collected as substrate for this work.

All tested effluents had a VFA composition including different acids, with acetic acid in the majority (Table 3.3.). The largest acids amounts are present in the BWW and PE, in which the acetic acid of

Table 3.2.: Antioxidant activity and total phenolic (TP) content

Effluents	Antioxidant activity (mmol TEAC)	DPPH Inhibition (%)	TP (gL^{-1})
PE	1.11 ± 0.01	80.9 ± 0.7	0.89 ± 0.00
OMW	0.80 ± 0.07	60.9 ± 3.0	3.12 ± 0.02
BWW	0.04 ± 0.07	6.7 ± 4.9	0.00 ± 0.00
I	0.18 ± 0.07	16.0 ± 4.8	0.00 ± 0.00

PE = piggery effluent; OMW = olive mill wastewater; BWW = brewery wastewater; I = inoculum; DPPH = 2,2-diphenyl-2-picrylhydrazyl hydrate

BWW almost duplicates the concentration of the latter (2.27 versus. 1.37 g mL^{-1}). In opposition, OMW presents the lowest VFA concentration and it indicates that the flow provided by the olive oil production is comparatively more preserved than the other two effluents.

Table 3.3.: Volatile fatty acids and pH

Effluents	Acetic acid (mgL^{-1})	Propionic acid (mgL^{-1} in acetic acid)	Isobutyric acid (mgL^{-1} in acetic acid)	Butiric acid (mgL^{-1} in acetic acid)	Total (mgL^{-1} in acetic acid)	pH
PE	1373.00	561.75	2157.74	1545.72	5638.21	7.27
OMW	213.00	64.04	110.41	159.48	546.93	5.09
BWW	2272.00	551.21	66.79	237.17	3127.18	5.10
I	1094.00	231.02	100.87	104.96	1530.85	7.43

PE = piggery effluent; OMW = olive mill wastewater; BWW = brewery wastewater; I = inoculum

3.2. First essay: anaerobic digestion with piggery effluent and olive mill wastewater

3.2.1. Biogas production

Biogas production was registered in all tested mixtures without any "lag" phase (see Figure 3.1.). The biogas production started immediately and a similar accumulated volume, around 120 mL, was observed in all units, elapsed 13 days. From then on and over the remaining experimental time, units containing 100%PE and 30%OMW+70%PE showed a similar behaviour, having provided the highest accumulated biogas amount (about 780 mL, Figure 3.1.) of all experiment.

Units containing 50%OMW+50%PE had maintained a stable period without producing biogas, for a period of about 30 days reaching a volume of 120 mL, approximately. After 30^{th} day, the assay was able to generate some gas until the end but not exceeding the mean value of 330 mL. This performance can be understood as the result of a late process (after day 30^{th}), in which the microorganisms were adapted to degrade polyphenols (from OMW). After that, the biogas producers (the methanogenic) were able to generate more gas. Comparatively, the gas production absence from the other essay, involving a volume participation of 80% of OMW (80%OMW+20%PE), suggests that, under the tested operating conditions, around 50% v/v the inhibitory influence of OMW starts to affect the biogas production in the blends.

The high standard deviation obtained in 50%OMW+50%PE essay (Figure 3.1.) results from a distinguished behaviour of one essay triplicate (cf. Material and Methods), in relation of the other two units. To illustrate the data, the biogas production is presented separately in the Figure 3.1.a. (unit 5 versus 4 and 6). The higher biogas production observed in unit 5, mainly at the end of experimental time, can be related to a new adaptation process of the microbial consortium that had developed in this digester.

Concerning biogas composition, the units containing 100%PE and 30%OMW+70%PE showed the highest methane concentrations (around 70% CH_4; Table 3.4.), being in agreement with the previous observations and indicating the presence of an active methanogenic archaea population in both assays. The value of 60% of methane in biogas, from 50%OMW+50%PE mixture, may result from an adaption process of the remaining consortium that maintained the capacity of convert the substrate and produce biogas. As expected, a very poor biogas was obtained in 80%OMW+20%PE, confirming the negative influence in the anaerobic consortium if a higher OMW proportions are included in the unit influent.

From the data recorded during the experiment it is possible to infer that OMW is a substrate with high inhibitory capacity against anaerobic digestion processes, only allowing small proportions (30% v/v) being mixed with PE. Using larger proportions in the blend (> 50%) results in deficient or non-biogas production.

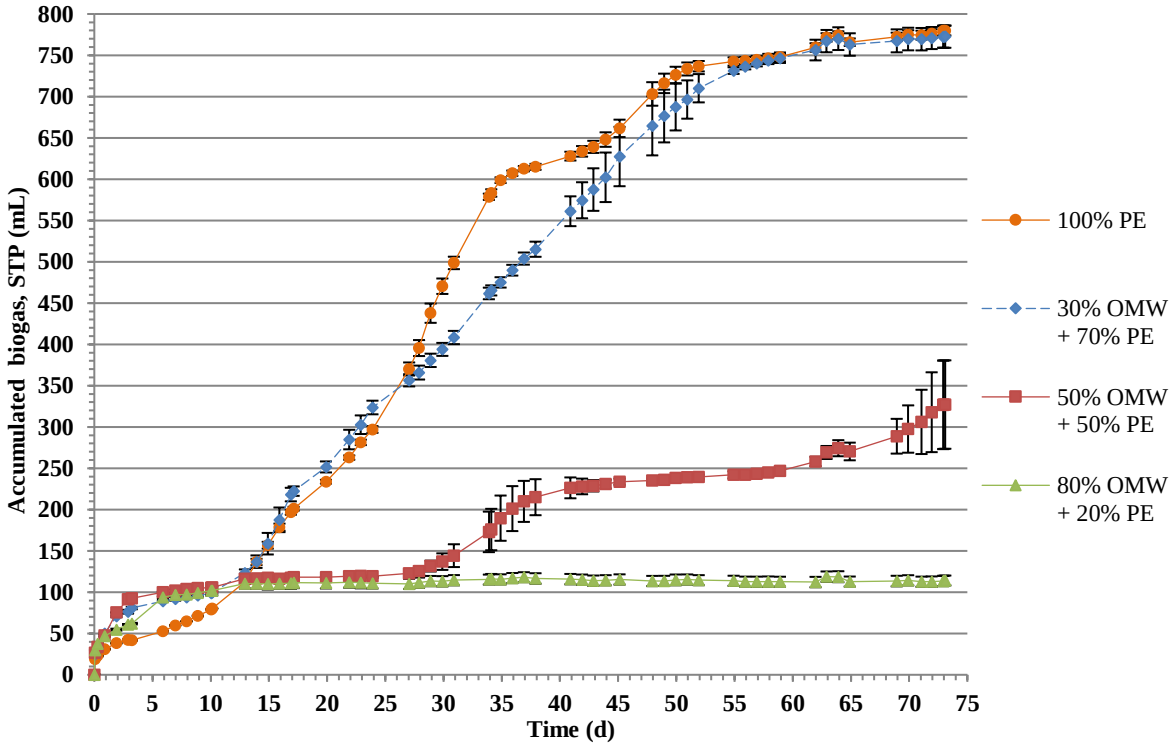

Fig. 3.1.: Biogas production of the first experiment, involving PE and OMW

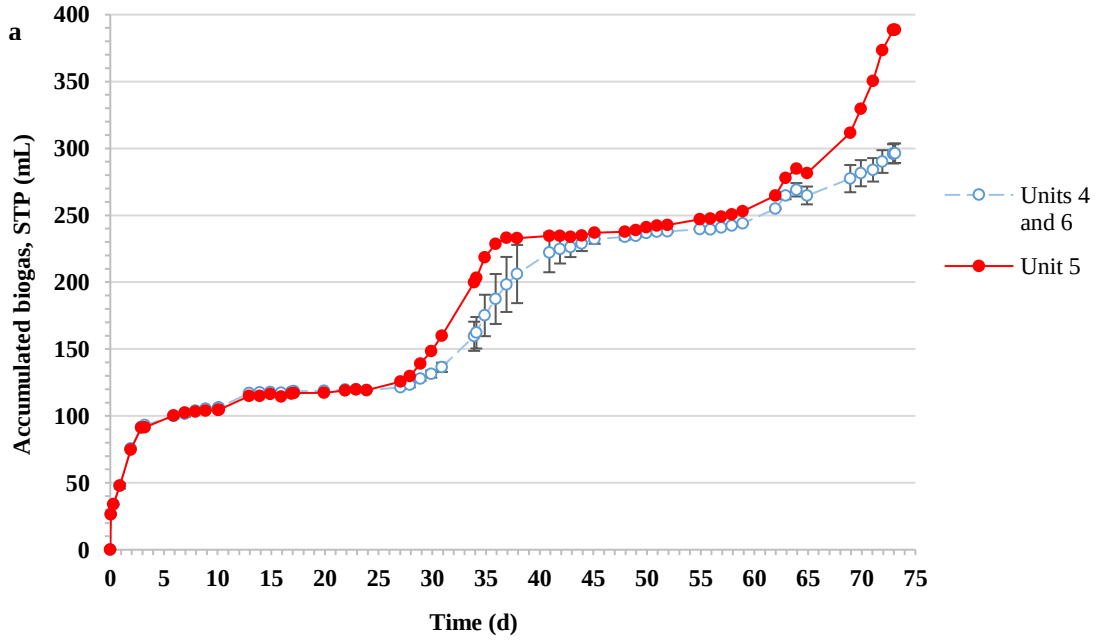

Fig. 3.1.a.: Biogas production of 50% OMW + 50% PE units

Table 3.4.: Biogas composition of the first experiment

Mixtures	Biogas (%, v/v)	
	CH4	CO2
100%PE	71.00 ± 0.27	29.00 ± 0.27
30%O+70%PE	70.27 ± 0.57	29.73 ± 0.57
50%O+50%PE	60.30 ± 6.68	39.70 ± 6.68
80%O+20%PE	5.57 ± 8.86	94.43 ± 8.86

3.2.2. Treatment capacity

As expected, the biggest removals of organic compounds belonged to 100%PE and 30%OMW+70%PE, the latter being better than the former, in these experimental conditions (62.5% versus 74.6% COD removal, respectively; Table 3.5.). On the opposite, 50%OMW+50%PE and 80%OMW+20%PE had eliminated less organic load, the latter having the lowest result overall (47.6% versus 28.9% COD, respectively). These results indicate a link between the amount of OMW present in the mixture and its influence over organic removal; on the last mixture, because it had the highest concentration of OMW, the inhibitory effects rendered the microorganisms unable to degrade organic compounds to form biogas. Concerning solids, 30%OMW+70%PE had the best results overall in removing volatile solids and total (21.0% in total and 35.6% of volatiles; see Table 3.6.). The 80%OMW+20%PE mixture removed approximately 21% of total solids, during the essay, but less in volatile (about 25%), suggesting that removing this type of solids was inhibited by OMW due to its toxic compounds. The 100%PE units removed almost 30% of volatile solids, but less in total, indicating presence of mineral solids not degradable by microorganisms.

Table 3.5.: Chemical oxygen demand (COD) of the first experiment

Mixtures	Initial COD (gL^{-1})	Final COD (gL^{-1})	Removal (%)
100%PE	93.2 ± 5.0	35.0 ± 7.1	62.5
30%O+70%PE	80.8 ± 2.5	20.5 ± 0.8	74.6
50%O+50%PE	77.3 ± 2.5	40.5 ± 2.4	47.6
80%O+20%PE	72.6 ± 0.8	51.6 ± 0.8	28.9

Concerning the polyphenols, it was observed an increase in concentration as the OMW volume rouse in the substrate, which was expected to occur (see Table 3.7.). The 100%PE and 80%OMW+20%PE mixtures showed the lowest removals and, despite the small initial phenolic concentrations of 100%PE, microorganisms did not show capacity of removing them. On the other side, the highest concentration of phenolic compounds in 80%OMW+20%PE mixture prevented the removal process due to high inhibitory effect of OMW. The 30%OMW+70%PE and 50%OMW+50%PE mixtures had the best results in removing the phenolic compounds (35% and 30%, respectively), indicating that their microbial consortia were able to degrade these kind of molecules. However, comparing the biogas volumes production, the 50%OMW+50%PE units needed about 25 days (shown on Figure 3.1.) to start to accumulate gas and, probably, to degrade part of the initial phenolic compounds present in higher

Table 3.6.: Total and volatile solids of the first experiment

Mixtures	Total Solids			Volatile Solids		
	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
100%PE	47.4 ± 0.8	39.6 ± 0.1	16.5	31.9 ± 0.6	22.5 ± 0.1	29.5
30%O+70%PE	39.6 ± 0.0	31.3 ± 0.7	21.0	27.8 ± 0.0	17.9 ± 1.1	35.6
50%O+50%PE	38.0 ± 0.1	31.9 ± 1.2	16.1	27.7 ± 0.0	21.6 ± 0.8	22.0
80%O+20%PE	32.8 ± 0.6	26.0 ± 0.8	20.7	25.4 ± 0.6	19.0 ± 0.5	25.2

concentration than in the 30%OMW+70%PE digesters (2.2 versus 1.7 g L^{-1}). The phenolic profiles for 100%PE (Appendices figures 1.a. and 2.a.) reveals that during the digestion some changes occurred, meaning that some phenolic compounds might be degraded and/or formed due to the reaction's conditions. Only one peak could be identified as being the 3-hydroxybenzoic acid, a phenolic monomer that can be formed by degradation of different compounds present in plants. The differences between the profiles of 30%OMW+70%PE (Appendices figures 2.b. and c.) from the original effluents suggests that some reactions occurred after mixing, either by degradation due to the new solution conditions or reacting with other compounds, altering the phenolic composition. The mixtures with higher OMW volumes also presented different profiles than the original effluents (data not shown), however it wasn't possible to obtain phenolic profiles after digestion.

Table 3.7.: Antioxidant activity and total phenolic (TP) content of the first experiment

Mixtures	Antioxidant Activity (mmol TEAC)		DPPH Inhibition (%)		TP		
	Initial	Final	Initial	Final	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
100%PE	1.11 ± 0.01	0.64 ± 0.10	80.9 ± 0.7	30.3 ± 8.3	0.89 ± 0.00	0.86 ± 0.00	3.37
30%O+70%PE	1.07 ± 0.01	0.39 ± 0.08	78.6 ± 0.3	9.7 ± 6.6	1.69 ± 0.00	1.10 ± 0.00	34.91
50%O+50%PE	0.99 ± 0.02	1.34 ± 0.03	73.7 ± 1.3	69.1 ± 1.7	2.18 ± 0.00	1.52 ± 0.00	30.28
80%O+20%PE	0.91 ± 0.01	1.35 ± 0.03	68.1 ± 0.2	69.5 ± 1.5	2.69 ± 0.00	2.50 ± 0.00	7.06

DPPH = 2,2-diphenyl-2-picrylhydrazyl hydrate

Regarding the antioxidant activity, there was not much difference between the mixtures values at the start of the essay, ranging between 0.9 – 1.1 mmol TEAC (see Table 3.7.). However, after anaerobic digestion, in 100%PE and 30%OMW+70%PE a decrease in antioxidant activity and inhibition was observed, suggesting a loss of compounds of interest together with other phenolic compounds during the anaerobic conversion process and through microorganisms' actions. In the case of the 50%OMW+50%PE units, this variation was not as pronounced, indicating that some of the compounds of interest found in the initial substrate were preserved in the digested material or, on the other hand, they were converted into others and compounds with higher antioxidant activity were released. The 80%OMW+20%PE behaviour concerning the variation of antioxidant activity and inhibition, after anaerobic digestion, is more pronounced and consistent with its diminishing removal capacity. The lack of phenolic compounds removal may result from the inhibition of the microbial consortia by the highest phenolic concentrations presents in the mixture containing the greatest OMW volume.

The highest concentrations of nitrogen compounds, both total and ammonium, in the feed substrate, present in the 100%PE units, decreased as the volume of OMW in the mixture was increased (Table 3.8.), as expected. Total nitrogen concentrations did not undergo major changes during digestion while the ammonium nitrogen parameter was subjected to increases as a result of the degradation compounds process, mainly proteins.

All essay mixtures presented neutral or almost neutral pH prior to anaerobic digestion, varying from 7.27 (100%PE) to 6.23 (80%OMW+20%PE), prior to anaerobic digestion (see Table 3.9.). At the end units had become more alkaline, indicating an effective removal of acidic compounds. The exception

Table 3.8.: Total and ammonium nitrogen of the first experiment

Mixtures	Total Nitrogen			Ammonia nitrogen		
	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)
100%PE	4900.0 ± 277.2	-	-	3206.0 ± 19.8	3864.0 ± 158.4	-20.5
30%O+70%PE	1831.2 ± 23.8	1845.2 ± 75.2	-0.8	1353.8 ± 112.9	2548.0 ± 297.0	-88.2
50%O+50%PE	1352.0 ± 4.0	1321.6 ± 23.8	2.2	827.4 ± 29.7	946.4 ± 11.9	-14.4
80%O+20%PE	579.6 ± 11.9	582.4 ± 39.6	-0.5	340.2 ± 13.9	296.8 ± 4.0	12.8

concerns 80%OMW+20%PE, in which the pH decreased (from pH 6.23 to 5.73; Table 3.9.), confirming the unsuitable operation of the process. The inhibition on microorganisms blocked the degradation of acid compounds throughout the essay. PE acted as buffer solution during the experiment due to the presence of high nitrogen concentration (Table 3.8.), counteracting the acidic nature of OMW. The presence of PE in 30%OMW+70%PE helps to maintain the medium pH in values appropriate to the development and maintenance of the anaerobic process despite the high VFA concentrations in the substrate (3.9 g L^{-1}). On the contrary, 80%OMW+20%PE presented a lower amount of acids than the former, but they also have a lower proportion of PE and, in addition, a high concentration of phenolic compounds that may have contributed to the mixture acidification.

Regarding VFA, 100%PE had removed most of its acids during the experiment (85%; Table 3.10.), including the butyric acid (1.5 g L^{-1}), confirming the presence of an active microbial population (bacteria and archaea) capable of converting these acids into CH_4. Concerning 30%OMW+70%PE mixture, more than half the initial VFA amount (56%) were removed, while on 50%OMW+50%PE, an increase in VFA concentration, except in acetic acid, was observed. The accumulation of VFA with a larger chain than acetic means some instability of the process, although it does not cause complete inhibition.

The recorded data suggests that the blending of OMW and PE in order to treat both effluents and generating biogas as by-product is feasible. However, it was only possible blending to use a small proportion of OMW (till 30% v/v) to produce methane in satisfactory amounts and avoiding its inhibitory effects at the same time. At higher volumes, the OMW might hinder the microbial population, affecting the treatment. Moreover, the experiment opens the possibility of using OMW as a substrate to produce CH_4 and provide its treatment, through removal of organic and toxic compounds removal by mesophilic anaerobic digestion.

Table 3.9.: pH values of the first experiment

Mixtures	Initial pH	Final pH
100%PE	7.27	8.05
30%O+70%PE	6.90	7.94
50%O+50%PE	6.66	7.25
80%O+20%PE	6.23	5.73

Table 3.10. Volatile fatty acids of the first experiment

Mix-tures	Acetic acid (mgL^{-1})		Propionic acid (mgL^{-1} in acetic acid)		Isobutyric acid (mgL^{-1} in acetic acid)		Butyric acid (mgL^{-1} in acetic acid)		Total IN (mgL^{-1} in acetic acid)	Re-moval (%)
	Initial	Final	Initial	Final	Initial	Final	Initial	Final		
100%PE	1373.00	688.00	561.75	117.54	2157.74	25.90	1545.72	0.00	5638.21	85.25
30%O+70%PE	2314.00	1190.00	1061.90	421.52	331.23	68.15	254.89	81.78	3962.02	55.54
50%O+50%PE	2175.00	2061.00	980.03	3153.27	288.97	926.89	239.90	534.32	3683.90	-81.21
80%O+20%PE	1801.00	757.00	553.65	109.43	316.23	38.17	121.31	423.91	2792.19	52.42

3.3. Second essay: anaerobic digestion with brewery wastewater and olive mill wastewater

3.3.1. Biogas production

In this experiment, several digesting mixtures, containing 50, 30 and 10% of BWW ceased their biogas production on day 6th (Figure 3.2.). The 70%BWW+I was the exception, showed an accumulated biogas of 66 mL, after elapsed 12 days (see Figure 3.2.). The recorded data evidence that the lack of biogas was

due to BWW being a diluted effluent. This implies that the microorganisms, present mainly in inoculum, hadn't much organic compounds to feed on and convert it to biogas, hence the accumulated production was below the mean value of 70 mL in all mixtures (Figure 3.2.). Additionally, it was possible to observe the toxic effects of OMW at different dilution levels. Only the units with the lowest OMW amount (20%) were able to accumulate biogas volumes higher than 20 mL, after about 20 of experimental days. On this operational condition, the high standard error presented results from the differential behaviour of one reactor (unit number 16) against the other two (units 17 and 18, Figure 3.2.a.). The best performance of unit 16 suggests that microbial population overcome the effect of inhibitory compounds

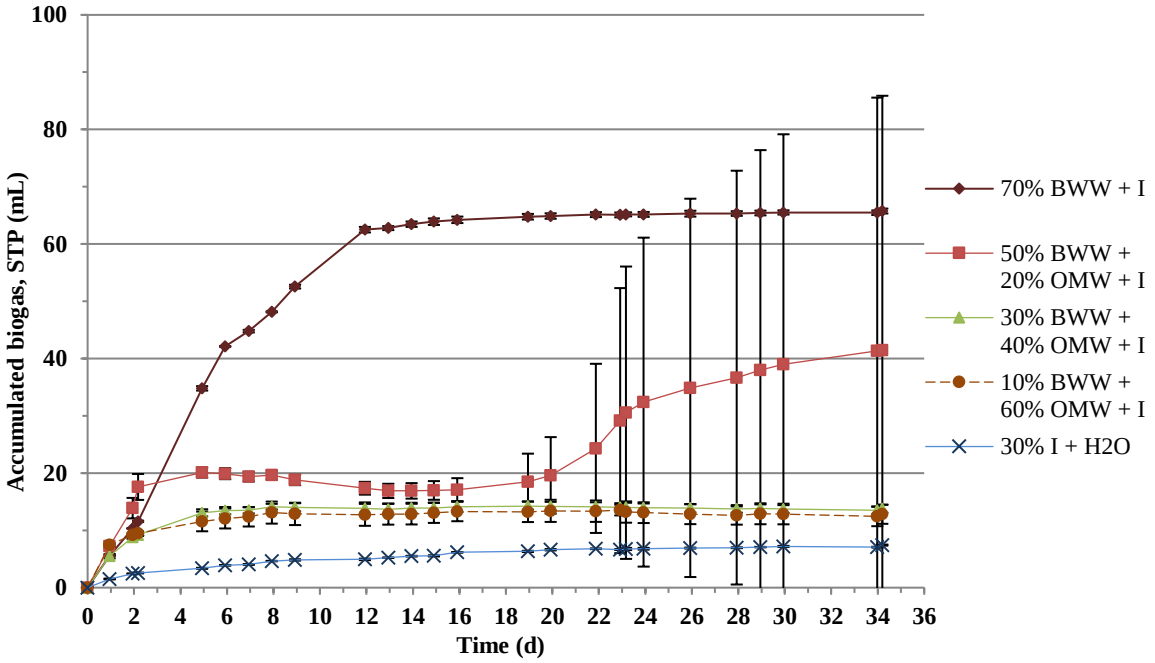

Fig. 3.2.: Biogas production of mixtures from the second experiment using OMW and BWW

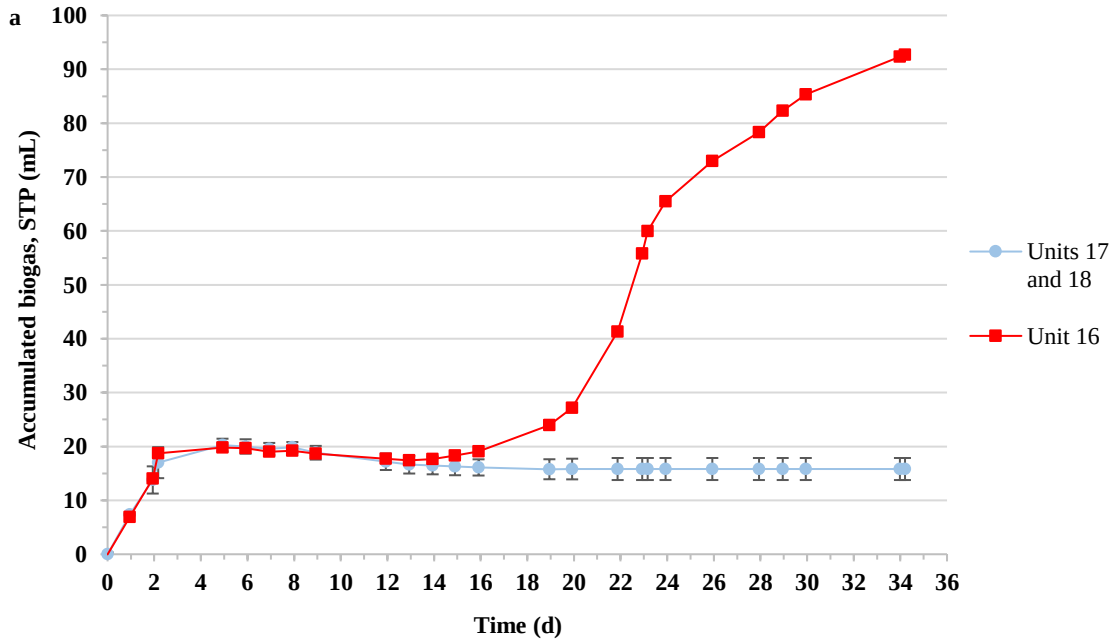

Fig. 3.2.a.: Biogas production of 50% BWW + OMW 20% + I units

present in OMW by adaptation to the operational conditions, recovering the capacity to produce more biogas.

All mixtures had produced low amounts of methane (less than 5% v/v; see Table 3.11.), the exceptions were 70%BWW+I and the control (30%I+H_2O). This suggests an inhibition of methanogenic archaea by OMW effluent, blocking the methane production. However, on 50%BW+20%OMW+I, the same unit which produced higher amounts of biogas also produced higher percentage of CH_4, detected on the second measurement (62.9% versus 6.4% obtained on first analysis; see Table 3.11.), meaning a better quality of biogas at the end of experiment. A possible cause for this is the adaptation of microbial population to the environment in the unit and the degradation of the inhibitory compounds from OMW, allowing the methanogenic population to regain the capacity to produce methane. Additionally, the two other units also increased their biogas quality, but in poor percentages (23.40% versus 3.05% CH_4; see Table 3.11.).

From the recorded data is possible to observe the negative effects of OMW during the anaerobic digestion, in conjunction with another more diluted effluent. This also implies that it isn't feasible blending OMW with a diluted effluent in order to be treated and producing methane.

Table 3.11.: Biogas composition of the second experiment

Mixtures	Biogas (%, v/v)			
	CH_4		CO_2	
	Day 14	Day 34	Day 14	Day 34
70%BW+I	79.23 ± 0.31	80.10 ± 0.27	20.77 ± 0.31	19.93 ± 0.32
50%BW+20%O+I	6.40a 3.05 ± 0.78b	62.90a 23.40 ± 0.78b	93.60a 96.95 ± 7.07b	37.10a 76.60 ± 7.07b
30%BW+40%O+I	1.57 ± 0.12	1.70 ± 0.10	98.43 ± 0.12	98.30 ± 0.10
10%BW+60%O+I	1.73 ± 0.32	1.53 ± 0.21	98.27 ± 0.32	98.47 ± 0.21
I+H2O	67.73 ± 0.75	72.13 ± 0.51	32.27 ± 0.75	27.87 ± 0.51

a = unit 16; b = units 17-18

3.3.2. Treatment capacity

The essay mixtures had low concentrations of organic load, as determined by COD (Table 3.12.). Additionally, minor changes were registered during the anaerobic digestion, with 70%BWW+I and control removing the organic compounds and the other mixtures with OMW increasing their concentration. The results further suggest OMW had a toxic effect over microbial populations, preventing the degradation of organic compounds to produce biogas.

Table 3.12.: Chemical oxygen demand (COD) of the second experiment

Mixtures	Initial COD (gL^{-1})	Final COD (gL^{-1})	Removal (%)
I+H2O	3.5 ± 0.4	2.3 ± 0.0	35.4
70%BW+30%I	7.0 ± 0.0	2.6 ± 1.2	63.5
50%BW+20%O+I	17.0 ± 0.4	20.2 ± 5.2a 21.1 ± 3.2b	-19.0a -24.0b
30%BWW+40%O+I	27.8 ± 2.7	30.8 ± 2.4	-10.7
10%BWW+60%O+I	36.5 ± 1.2	40.2 ± 1.6	-15.7

a = unit 16; b = units 17-18

Concerning solids removal, 70%BWW+I and 50%BWW+20%OMW+I (units 17 and 18, only) had removed the most amount of solids during the experiment (see Table 3.13.). The latter essay mixture had removed more total solids but slightly less volatile solids than the former (see Table 3.13.). On the

others mixtures, the low amount of solids removed again suggests an inhibition of OMW on microbial population, hindering the biogas production from the degraded solids.
As expected, the essay mixtures with OMW in their composition had high concentrations of phenolic compounds (Table 3.14.). Moreover, OMW toxicity might have inhibited the microbial population from degrading the phenolic compounds during the experiment. Having lower proportion of the effluent, the units from 50%BWW+20%OMW+I mixture removed the highest amount of phenolic compounds

Table 3.13.: Total and volatile solids of the second experiment

Mixtures	Total Solids			Volatile Solids		
	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
I+H2O	2.4 ± 0.1	2.4 ± 0.1	0.0	1.7 ± 0.0	1.6 ± 0.1	5.9
70%BW+I	4.8 ± 0.0	3.9 ± 0.2	18.8	2.7 ± 0.1	1.8 ± 0.1	33.3
50%BW+20%O+I	9.9 ± 0.1	9.6a 7.8 ± 1.2b	3.0a 21.2b	7.1 ± 0.0	6.7a 5.1 ± 1.2b	5.6a 28.2b
30%BW+40%O+I	15.4 ± 0.0	14.0 ± 1.1	9.1	12.1 ± 0.0	10.3 ± 0.8	14.9
10%BW+60%O+I	21.3 ± 0.1	19.2 ± 0.0	9.9	17.0 ± 0.7	14.6 ± 0.6	14.1

a = unit 16; b = units 17-18

during the experiment, but less than half the initial amount (38.5% of maximum removal; Table 3.14.). Control and 70%BWW+I had traces amounts of phenolic compounds because of BWW and the inoculum's nature, so it wasn't observed any removal of phenolic compounds. The electropherograms obtained for this essay are from 30%BWW+40%OMW+I and 10%BWW+60%OMW+I mixtures (Appendices figures 3.a. and b.). Both present a poor phenolic profile, despite the high concentrations, and different from the original effluents' profiles, possibly influenced by the inoculum. For the other mixtures it was not possible to obtain a phenolic profile.
The mixtures had low nitrogen concentration due to the effluents lack of these molecules (Table 3.15.). Apart from 70%BWW+I, the mixtures didn't undergo major changes during the experiment. A possible cause might be the microbial population was unable to process the molecules, in same way as wasn't able to degrade the organic load. On the opposite, an increase in ammonium concentration in 70%BWW+I was observed (from 81.2 mg L^{-1} to 123.2 mg L^{-1}; see Table 3.15.), indicating that part of the nitrogen compounds were degraded.

Table 3.14.: Antioxidant activity and total phenolic (TP) content of the second experiment

Mixtures	Antioxidant activity (mmol TEAC)		DPPH Inhibition (%)		TP		
	Initial	Final	Initial	Final	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
I+H2O	0.03 ± 0.05	0.04 ± 0.08	5.4 ± 3.3	0.0 ± 6.6	0.00 ± 0.00	0.00 ± 0.00	0.00
70%BW+I	0.19 ± 0.21	0.06 ± 0.06	0.0 ± 17.2	0.0 ± 4.6	0.01 ± 0.00	0.01 ± 0.00	0.00
50%BW+20%O+I	0.89 ± 0.02	1.17 ± 0.03b	63.2 ± 1.3	72.9 ± 2.8b	0.65 ± 0.05	0.47 ± 0.00a 0.40 ± 0.00b	27.69a 38.46b
30%BW+40%O+I	0.65 ± 0.06	1.47 ± 0.02	46.8 ± 4.3	77.1 ± 1.2	0.98 ± 0.00	0.87 ± 0.00	11.22
10%BW+60%O+I	0.59 ± 0.09	1.46 ± 0.01	43.3 ± 5.8	76.3 ± 0.4	1.54 ± 0.00	1.37 ± 0.01	11.04

a = unit 16; b = units 17-18; DPPH = 2,2-diphenyl-2-picrylhydrazyl hydrate

The mixtures, after anaerobic digestion, had their pH decreased except 70%BWW+I and one essay unit from 50%BWW+20%OMW+I (Table 3.16.). Mixtures who had OMW were slightly acidic at the start, with 10%BWW+60%OMW+I being the most acidic. After the experiment it was observed that the essay mixtures had their pH below 5.5, except the ones mentioned earlier. These results suggest the

microorganisms were not capable of degrading acid compounds from OMW. A possible cause might be the OMW blocking the activity of microbial population with the acids and toxic compounds and, possibly, the non-existence of buffer capacity due to the lack of nitrogen compounds.

Table 3.15. Total and ammonium nitrogen of the second experiment

Mixtures	Total nitrogen			Ammonium nitrogen		
	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)
I+H2O	170.8 ± 19.8	-	-	102.2 ± 2.0	110.6 ± 2.0	-8.2
70%BW+I	184.8 ± 23.8	201.6 ± 7.9	-9.1	81.2 ± 0.0	123.2 ± 0.0	-51.7
50%BW+20%O+I	224.0 ± 0.0	-	-	96.6 ± 2.0	86.8 ± 4.0a 92.4 ± 4.0b	10.1a 4.3b
30%BW+40%O+I	252.0 ± 7.9	238.0 ± 4.0	5.6	93.8 ± 2.0	88.2 ± 2.0	6.0
10%BW+60%O+I	257.6 ± 15.8	254.8 ± 4.0	1.1	92.4 ± 0.0	78.4 ± 0.0	15.2

a = unit 16; b = units 17-18

Concerning VFAs, all mixtures with OMW had increased their VFAs' concentration, apart from unit number 16 of 50%BWW+20%OMW+I essay mixture (see Table 3.17.). The 70%BWW+I units had removed the most in this experiment, mainly acetic and propionic acid, suggesting an active methanogenic population. However, it was observed an increase in 4-carbon chain acids' concentration (from 98.14 mg L^{-1} to 154.56 mg L^{-1} in butyric acid, and from none to 106.49 mg L^{-1} in isobutyric acid; see Table 3.17.), also indicating a possible conversion of some of acetic acid into the latter forms. In 50%BWW+20%OMW+I's unit 16, the acetic acid was almost completely removed, however, it was observed a concentration increase in other acids. This could suggest that, when the methanogenic population was inhibited, the acetic acid was converted into other VFAs. After being no longer inhibited, the methanogenic archaea, as shown on graph 3.4.a., began to use acetic acid to produce biogas.

The essay mixtures which had OMW in their composition presented antioxidant activity, ranging between 0.6 – 0.9 mmol TEAC (Table 3.14.). The same mixtures increased these values throughout the experiment, to above 1.1 mmol TEAC, with 30%BWW+OMW40%+I having the highest value. Phenolic compounds from OMW in high concentrations might be the possible cause for this activity. Control and 70%BWW+I didn't show any activity because there was no significant activity from the BWW and inoculum themselves, as shown on Table 3.2.

From the recorded data it is possible to infer that diluting OMW with a low organic load effluent in order to produce methane through mesophilic anaerobic digestion is not viable, because even on mixture with highest dilution (50%BWW+20%OMW+I), the inhibitory effects of OMW on methanogenic population were still present despite the low produced methane.

Table 3.16.: pH values of the second experiment

Mixtures	pH	
	Initial	Final
I+H2O	7.59	7.43
70%BW+I	6.70	7.17
50%BW+20%O+I	6.30	6.62a 5.15b
30%BW+40%O+I	6.14	5.01
10%BW+60%O+I	5.97	4.80

a = unit 16; b = units 17-18

Table 3.17. Volatile fatty acids of the second experiment

Mixtures	Acetic acid (mgL^{-1})		Propionic acid (mgL^{-1} in acetic acid)		Isobutyric acid (mgL^{-1} in acetic acid)		Butyric acid (mgL^{-1} in acetic acid)		Total IN (mgL^{-1} in acetic acid)	Removal (%)
	Initial	Final	Initial	Final	Initial	Final	Initial	Final		
I+H2O	1072.00	891.70	187.25	79.57	25.90	73.78	80.42	102.92	1365.57	15.93
70%BW+ I	1245.00	134.90	265.88	72.14	0.00	106.49	98.14	154.56	1609.02	70.91
50%BW+ 20%O+I	1354.00	93.60a 1649.30b	253.72	267.69a 339.57b	58.61	149.52a 201.30b	132.22	474.85a 948.64b	1798.55	45.20a -74.52b
30%BW+ 40%O+I	1277.00	2058.40	201.84	274.97	80.42	110.55	91.33	518.98	1650.59	-79.51
10%BW+ 60%O+I	458.00	1466.40	57.55	159.63	61.34	0.00	14.99	243.57	591.89	-215.86

a = unit 16; b = units 17-18

3.3.3. Microscopy and pigment analysis

During the experiment, 70%BWW+I mixture presented reddish deposits in the medium. According to the literature, the colour is an indication of purpur non-sulfur bacteria (PNSB) (Soto-Feliciano *et al.*, 2010 and Zhang *et al.*, 2002). After the anaerobic digestion, a sample was taken and analysed under light microscopy and, as shown in Figure 3.3.a., reddish clusters were detected. The absorbance maxima of whole cells were found at 862, 806, 592, 528 and 490 nm in the absorption spectrum (Figure 3.3.b). These indicate the presence of bacteriochlorophyll a and carotenoids pigments of the spirilloxantin series, which are characteristic of PNSB (Okubo *et al.*, 2006 and Soto-Feliciano *et al.*, 2010).

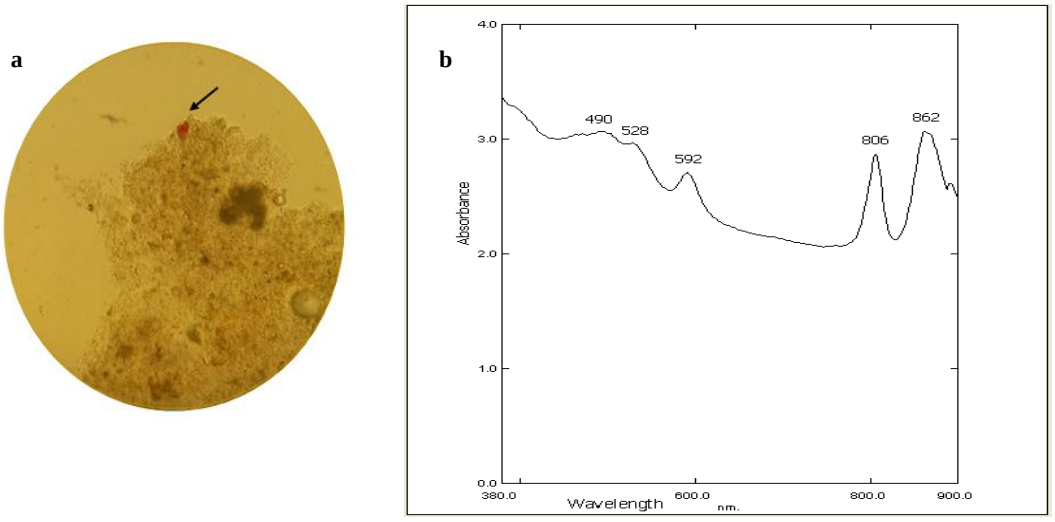

Fig. 3.3.: A liquid sample of culture medium analysed under light microscopy (amplification 400x) (a) and absorption spectrum of the whole cells sample (b). In (a), the black arrow points to the red cluster that contains pigments. In (b), the wavelength (nm) of absorption maxima are shown on top of each peak.

3.4. Third essay: anaerobic digestion with brewery wastewater and swine wastewater

3.4.1. Biogas production

During the first phase of this essay (HRT of 5.7 days, loading rate of 5.2 kg COD m^{-3} d^{-1}), the reactor produced between 0.4 L L^{-1} d^{-1} and 1.2 L L^{-1} d^{-1} of biogas. As shown on the Figure 3.4., the variations of biogas production were caused by not feeding the reactor with substrate during the weekends, hence the biogas production breakdowns. The obtained biogas was mostly composed by methane (between 63% and 77.8%, Figure 3.4. and Table 3.18.). On second phase (HRT of 3.0 days), there was an increase in biogas production (between 1.1 L L^{-1} d^{-1} and 2.3 L L^{-1} d^{-1}), suggesting that the microbial consortia were able to support the organic loading rise (10.0 kg COD m^{-3} d^{-1}). The methane content had slightly increased to 79.5% v/v (see Table 3.18.), indicating a quality improvement. On the last phase (HRT of 1.0 days, loading rates of 33.6 kg COD m^{-3} d^{-1}), the biogas production increased even further (between 2.4 L L^{-1} d^{-1} and almost 3.0 L L^{-1} d^{-1} per day), maintaining the biogas quality (79.5% v/v CH_4; Table 3.18.) and indicating that this hybrid reactor was capable of supporting high loads of organic-rich substrate and still being able to produce high volumes of biogas without suffering any washout event.

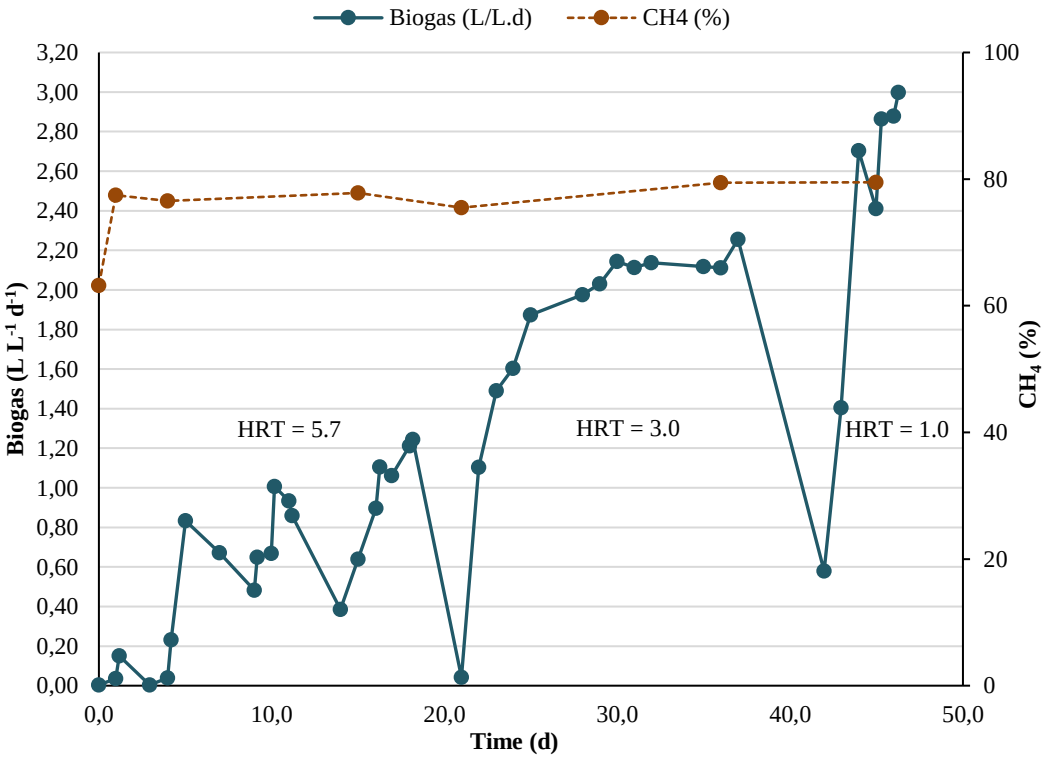

Fig. 3.4.: Biogas production and percentage of methane from third experiment. HRT = hydraulic retention time

Table 3.18.: Biogas composition of the third experiment

Biogas		
HRT	CH4 (%, v/v)	CO2 (%, v/v)
5.7	77.8	22.2
3.0	79.5 ± 0.1*	20.6 ± 0.1 *
1.0	79.5	20.5

HRT = hydraulic retention time; *mean value of two measurements of the same day

3.4.2. Treatment capacity

The decrease of COD concentration during the anaerobic process of the first phase of the experiment (see Table 3.19.) indicates that the digestion of the substrate was taken place, under the tested experimental conditions, as expected. However, the digested COD from the second phase was much higher (26.4 g L^{-1}, compared to 14.4 g L^{-1} from the first phase), contradicting the biogas production

Table 3.19.: Chemical oxygen demand (COD) of the third experiment

HRT	Initial COD (gL^{-1})	Final COD (gL^{-1})	Removal (%)	Loading rate (kg COD m^{-3} d^{-1})
5.7	29.9 ± 0.9	14.4 ± 0.0	51.8	5.2
3.0	29.9 ± 0.9	26.4 ± 0.0	11.7	10.0
1.0	33.6 ± 2.6	25.0 ± 0.9	25.6	33.6

HRT = hydraulic retention time

graph curve obtained. A possible explanation for that could be some organic particles and flocks that came out from the reactor, due to high substrate influx, and the reactor wasn't able to digest it in time. The COD of the digested from the third phase (HRT = 1.0 days) was similar to the latter, and possibly with similar causes, with the addition that in this last phase the reactor had efficiently produced more biogas and with a good quality, as mentioned before (Figure 3.4. and Table 3.19.).

Regarding solids, the reactor had removed more on the second phase of the experiment (54.2 % in TS, and 67.6% in VS, see Table 3.20.), suggesting that the microbial population had converted those solids into biogas. On the other two phases of this experiment, the less solids removal could be related with the high influx flow of the substrate on the latter phase, in which some solids might have been dragged out from the reactor, on the former phase the inorganic solids might be the cause for the low removals.

Concerning total phenolic content, the amount present in the substrate was below than 0.40 g L^{-1} (see Table 3.21.), due to low concentrations derived from PE and BWW. The microbial consortia inside the reactor was able to remove the same amount of phenolic compounds with the exception of the last phase, in which only 0.03 g L^{-1} were removed (Table 3.21.). Despite the initial low concentration, the recorded results showed that the microorganisms were able to eliminate the phenolic compounds, except on the last phase possibly because of high substrate influx. The electropherograms of the substrate before and after digestion are different from the original effluents, but they present poor phenolic profiles and identification of these compounds was not possible (Appendices figures 4.a. and b.).

Table 3.20.: Total and volatile solids of the third experiment

HRT	Total Solids			Volatile Solids		
	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
5.7	22.5 ± 1.3	20.8 ± 0.2	7.6	14.8 ± 1.0	11.6 ± 0.1	21.6
3.0	22.5 ± 1.3	10.3 ± 0.1	54.2	14.8 ± 1.0	4.8 ± 0.2	67.6
1.0	22.5 ± 0.2	20.3 ± 0.5	9.8	14.1± 0.2	12.2 ± 0.4	13.5

HRT = hydraulic retention time

Concerning nitrogen compounds, there were some observations: the nitrogen concentration decrease during the second phase of the experiment suggests protein degradation by the microorganisms inside the reactor (Table 3.22.). Also, the ammonium nitrogen concentration increase during the first and third phase of the experiment implies the same, despite the minimal difference before and after. In other results there are no significant changes in nitrogen compounds during the essay.

Table 3.21.: Antioxidant activity and total phenolic (TP) content of the third experiment

HRT	Antioxidant activity (mmol TEAC)		DPPH Inhibition (%)		TP		
	Initial	Final	Initial	Final	Initial (gL^{-1})	Final (gL^{-1})	Removal (%)
5.7	0.85 ± 0.04	0.23 ± 0.08	60.5 ± 3.0	0.0 ± 6.6	0.37 ± 0.00	0.23 ± 0.00	37.84
3.0	0.85 ± 0.04	0.41 ± 0.07	60.5 ± 3.0	12.6 ± 4.1	0.37 ± 0.00	0.23 ± 0.00	37.84
1.0	1.11 ± 0.05	0.81 ± 0.08	55.0 ± 3.2	37.0 ± 4.7	0.36 ± 0.00	0.33 ± 0.00	8.33

HRT = hydraulic retention time; DPPH = 2,2-diphenyl-2-picrylhydrazyl hydrate

Table 3.22.: Total and ammonia nitrogen of the third experiment

HRT	Total nitrogen			Ammonium nitrogen		
	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)	Initial (mgL^{-1})	Final (mgL^{-1})	Removal (%)
5.7	1047.2 ± 7.9	1086.4 ± 23.8	-3.7	747.6 ± 7.9	753.2 ± 4.0	-0.7
3.0	1047.2 ± 7.9	823.2 ± 15.8	20.5	747.6 ± 7.9	702.8 ± 4.0	6.0
1.0	-	-	-	786.8 ± 4.0	802.2 ± 17.8	-2.0

HRT = hydraulic retention time

The pH of substrate was neutral, due to PE counteracting the acidity of BWW. During the experiment, the pH of the reactor's effluents was slightly higher than the substrate, but still considered neutral, except only the first phase (see Table 3.23.). This suggests that acidic compounds of substrate, possibly VFAs, were degraded (evidenced by the nitrogen and VFA concentrations on Tables 3.22. and 3.24.). On the substrate was observed a high concentration of acetic and propionic acid, the first might have come from BWW and the latter from PE (through degradation of isobutyric and butyric acids; see Table 3.3.). A decrease in concentration on all VFAs was observed in all phases of the experiment, suggesting the microbial community, especially hydrogenotrophic bacteria, were capable of degrading the more complex acids into acetic acid while the methanogenic archaea population converted those into methane. It was on second phase that the reactor had better results at eliminating VFAs (95.4 % removal, see Table 3.24.), implying better efficiency of the reactor in this parameter. Also, the lower removal on the last phase (when HRT = 1.0 days) (64.4%) might be caused by high substrate influx, during which the microbial population hadn't the capacity to degrade the VFAs as well as in other phases.

About antioxidant activity, the results showed an activity loss of the substrate during the reactor process, suggesting some loss of the compounds of interest through anaerobic conversion processes and

Table 3.23.: pH values of the third experiment

HRT	pH	
	Initial	Final
5.7	7.00	8.13
3.0	7.00	7.86/7.95
1.0	7.38	7.80

HRT = hydraulic retention time

Table 3.24.: Volatile fatty acids of the third experiment

HRT	Acetic acid (mgL^{-1})		Propionic acid (mgL^{-1} in acetic acid)		Isobutyric acid (mgL^{-1} in acetic acid)		Butyric acid (mgL^{-1} in acetic acid)		Total IN (mgL^{-1} in acetic acid)	Removal (%)
	Initial	Final	Initial	Final	Initial	Final	Initial	Final		
5.7	2627.00	237.00	1079.73	254.73	234.45	25.69	294.42	17.54	4235.60	87.37
3.0	2627.00	152.70	1079.73	20.09	234.45	9.72	294.42	12.94	4235.60	95.38
1.0	1852.30	586.50	775.55	341.42	217.38	92.27	233.54	75.40	3078.80	64.41

HRT = hydraulic retention time

microorganisms' actions (see Table 3.21.). Also, during the third phase, the antioxidant activity was higher, meaning that the compounds with this activity were preserved or less degraded.
From the recorded data, an under the experimental conditions, it is possible to infer that the reactor worked more efficiently on the second phase. The exception was on COD parameter, which was better in the first phase.

3.4.3. Reactor profile

From the data obtained from the samples, shown on Figure 3.5., it is possible to observe differences between the samples. The second sample (at 14.5 cm of height) had the highest concentrations which decreased in the next sections (i.e. in the upper parts of the reactor), indicating a removal of the organic compounds from this point up through the reactor until the exit point. Between the point of entry and the first sample the VFAs concentration diminished significantly and, as shown on Table 3.25., in all acids, most noticeably in acetic acid (from 2627 mg L^{-1} to 173 mg L^{-1}). Throughout the reactor, the concentrations of each acid had changed but not as significant as in the lower part. The pH increased from the entry point until where the first sample was collected (i.e. at 14.5 cm of height), remaining relatively stable throughout the reactor (see Table 3.25.). The recorded data suggests a stratification inside the reactor, in which the lower layers might be where the majority of methanogenic and acetogenic microorganisms are located and act on the material in digestion, and possibly where most of biogas is produced. It also indicates a possible sludge bed formation in the same location, hinted by the increase in organic compounds and solids. On the upper layers is where the microorganisms capable of degrading complex molecules are located, together with some acetogenic and methanogenic populations; both these populations might produce biogas from the previous digested substrate, partially degraded.

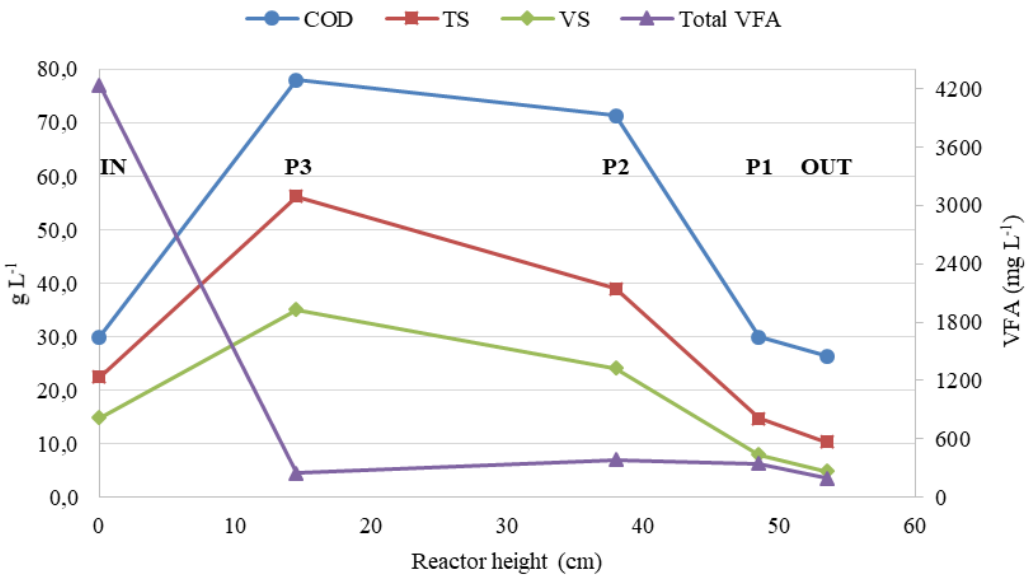

Fig. 3.5.: Characteristics of reactor's profiles. COD = Chemical oxygen demand; TS = total solids; VS = volatile solids, VFA = volatile fatty acids

Table 3.25.: Volatile fatty acids and pH of reactor's profiles

Reactor height (cm)	Acetic acid (mgL^{-1})	Propionic acid (mgL^{-1} in acetic acid)	Isobutyric acid (mgL^{-1} in acetic acid)	Butyric acid (mgL^{-1} in acetic acid)	pH
0.0 (IN)	2627.00	1079.73	234.45	294.42	7.00
14.5 (P3)	173.40	35.11	16.20	20.81	7.86
38.0 (P2)	187.00	43.69	88.55	68.35	7.94
48.5 (P1)	131.60	59.99	58.27	95.84	7.97
53.5 (OUT)	152.70	20.09	9.72	12.94	7.95

3.5. Microbial characterization by molecular analysis

3.5.1. DNA extraction

Samples of PE and OMW contained high organic load and phenols composition that compromise extraction of genomic DNA in terms of quantity and quality. Several trials were attempted in order to improve DNA extraction. The results were resumed in Table 3.26. The better results were obtained with trial D and the DNA extraction for sequencing proceeded using trial D, and later with trial E for samples containing OMW in their composition. Unfortunately, it was not possible to isolate DNA from raw OMW samples in quantity and quality for the sequencing, using the trials mentioned on the Table 3.26. According to the results obtained for best biogas/methane production, 12 samples were selected for microbial characterization through molecular analysis. The concentration and quality (A260/280) of extracted genomic DNA is shown in Table 3.27. The sample from the first phase (HRT 5.7) failed library preparation and was excluded from the sequencing run by StabVida.

Table 3.26.: Effect of the change in conditions of DNA extraction (trials A-E) on the purity (A260/A280) and decontamination yield (A260/A230)

Trial	Sample	DNA concentration ($ng\ \mu l^{-1}$)	A260	A280	A260/280	A260/230
A	PE	236.6	4.731	2.848	1.66	0.92
B	PE	109.3	2.187	1.257	1.74	0.70
C	OMW	418.2	8.364	8.377	1.00	0.41
D	Inoculum	234.7	4.694	2.693	1.74	1.26
E	50%BWW+20%OMW+30%Inout	66.3	1.326	1.059	1.25	0.36

Table 3.27.: DNA quantification (Qubit method) of samples sent for sequencing

Sample	DNA Concentration (ng μL^{-1})	A260/280
I	44.00	1.7
BWW	33.00	1.9
100% PE out	3.06	1.3
30%OMW+70%PE in	32.20	1.5
30%OMW+70%PE out	6.32	1.2
70%BWW+I in	11.30	1.5
70%BWW+I out	2.88	1.3
40%PE+60%BWW	22.40	1.3
HRT 5.7	Too low	1.3
HRT 3.0	16.96	1.4
HRT 1.0	3.30	1.3
PE	60.00	1.5

3.5.2. Next generation sequencing

After Next Generation Sequencing, the samples generated 507976 to 691834 raw sequence reads and 5322 OTUs were identified. Relative abundance of bacterial and archaea groups was determined in terms of a percentage of the total number of sequences in a sample.

3.5.2.1. Effluents and inoculum

The effluents which were studied for microorganisms' diversity had their results presented on Figure 3.6.a. As expected, there was a predominance of Bacteria domain in all of them (above 99%), being the total of microorganisms in BWW.

Related to Bacteria domain, a diverse population was found in all effluents (Figure 3.6.b.). However, these populations belonged mostly to phyla Proteobacteria (54.6%), Firmicutes (65.1%) and Bacteroidetes (53.2%) on Inoculum, PE and BWW, respectively. Most of Proteobacteria were Pseudomonadales (39.8%, Appendices figure 6.f.), in Firmicutes were Clostridiales (57.1%, Appendices figure 6.c.) and in Bacteroidetes were Bacteroidales (53.2%, Appendices figure 6.a.) only.

Proteobacteria also appears on both effluents in minor percentages (7.0% on PE and 16.6% in BWW, in relative abundance). Additionally, Firmicutes and Bacteroidetes also appeared in opposite effluents (respectively BWW and PE) and Chloroflexi appears on Inoculum, as sub-dominant phylum with 18.4% relative abundance, with Anaerolineales making the most of the phylum (Appendices figure 6.b.). Other phyla were detected, but due to their low percentages (between 1% and 10%) they weren't considered in this discussion.

For the Archaea domain, *Methanobrevibacter* was the predominant genus on PE (72.1% in relative abundance, Figure 3.6.c.), presenting other populations in much smaller percentages (less than 7%). The only found in Inoculum sample was *Methanosaeta*, in what could be explained by the specific environment of a wastewater treatment plant, where this microorganism was already adapted. No populations were detected in BWW belonging to Archaea domain.

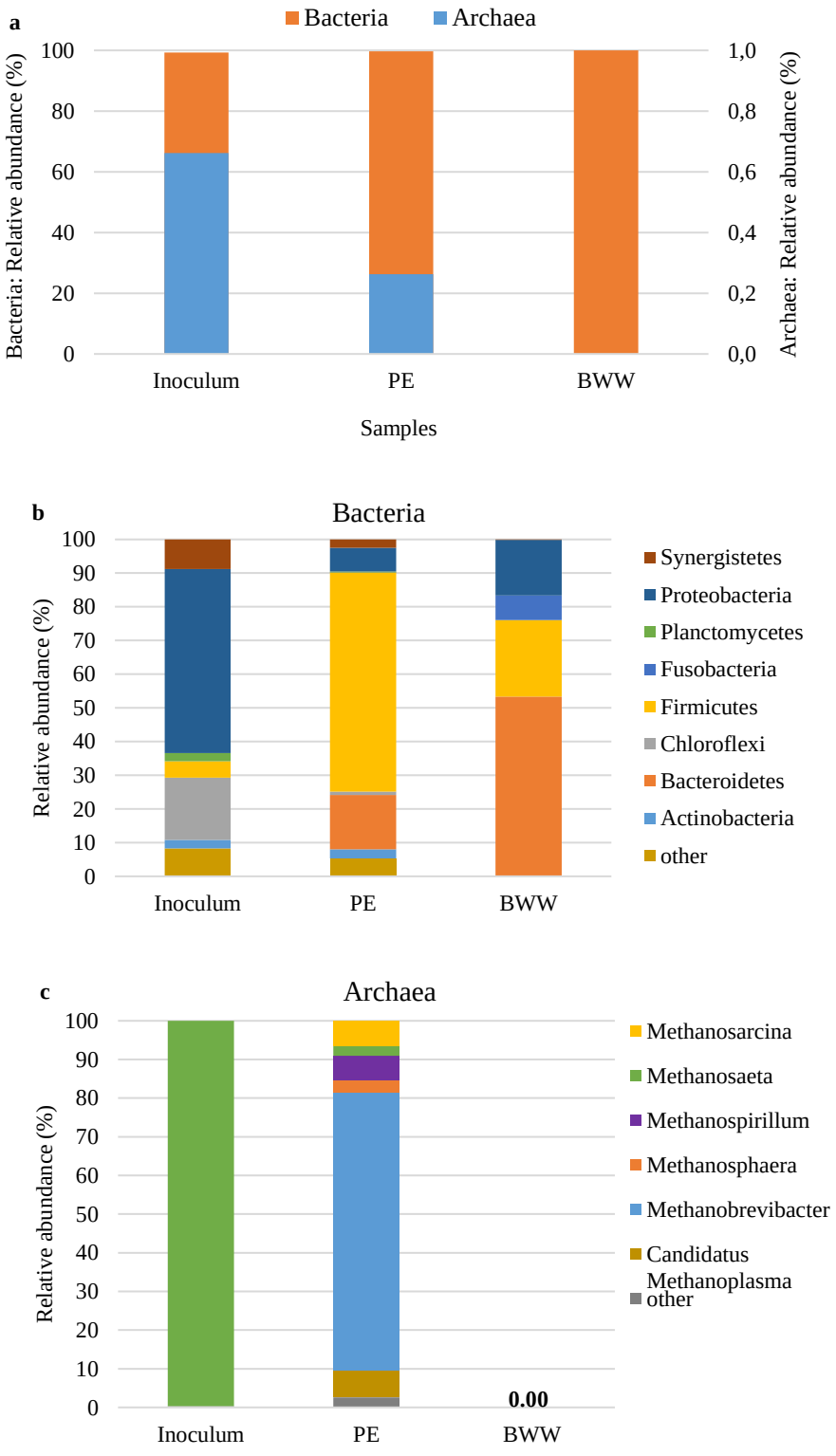

Fig. 3.6.: Relative abundance of Bacteria and Archaea domains (a), and their respective phyla (b) and genera (c), from the effluents and inoculum

3.5.2.2. First essay

From the first essay, only 100%PE and 30%OMW+70%PE (in and out) mixtures were studied for their biodiversity due to their high biogas production, mentioned earlier. The Figure 3.7.a. shows a predominance of microorganisms from Bacteria domain in both mixtures, while the presence of Archaea had diminished during the anaerobic digestion process.

Considering the effluent itself, 100% PE had most of its population belonging to phylum Firmicutes (65.1%), which predominance maintained during the whole experiment (Figure 3.7.b). Same situation was observed in 30%OMW+70%PE, with Bacteroidetes being the sub-dominant phylum, and having close representation to the former phylum at the start of the essay (30.6% versus 44.7% of Firmicutes, Figure 3.7.b). At the end of digestion, the Firmicutes' populations had a high percentage increase in both essay mixtures (from 65% to 94% in 100%PE, and from 45% to 90% in 30%OMW+70%PE, Figure 3.7.b), mostly represented by Clostridiales (85.2% in 100%PE, and 61.9% in 30%OMW+70%PE, Appendices figure 6.c.). The other populations, more specifically Bacteroidetes, became almost non-existent, suggesting that these two phyla might compete for the same resources and energy from degrading polysaccharides, proteins and lipids, according to Gannoun *et al.*, 2016 and Wang *et al.*, 2017. Also, the high metabolic versatility of Firmicutes suggests that cellulose residues from PE and other complex organic compounds present in OMW were degraded to form the acids (Buhlmann *et al.*, 2019; Nelson *et al.*, 2011 and Wang *et al.*, 2018). This metabolism could suggest a constant supply of acetate (also from aminoacids metabolized by Bacteroidetes; Gannoun *et al.*, 2016) and H2 to methanogens, according to Gannoun *et al.*, 2016, Treu *et al.*, 2019 and Wang *et al.*, 2017. Other phyla weren't considered in this discussion due to their low presence (in between 1% - 10%).

In relation to Archaea (Figure 3.7.c), it was observed substantial changes during anaerobic digestion. On 100%PE, *Methanosarcina* became the predominant population (from 6.5% to 89.6%) and *Methanobrevibacter* population decreased (from 72.1% to 9.87%). On 30%OMW+70%PE *Methanobrevibacter* and *Mehanosarcina* became predominant after the experiment, while other Archaean genera had, almost or totally, disappeared, notably *Candidatus Methanoplasma*. The presence of *Methanosarcina* in the two mixtures suggests methane formation mainly from acetate, according to Cho *et al.*, 2013, Wang *et al.*, 2017 and Wang *et al.*, 2018. Due to being detected with low percentages (below 10%), other genera present on the graph weren't considered for the discussion.

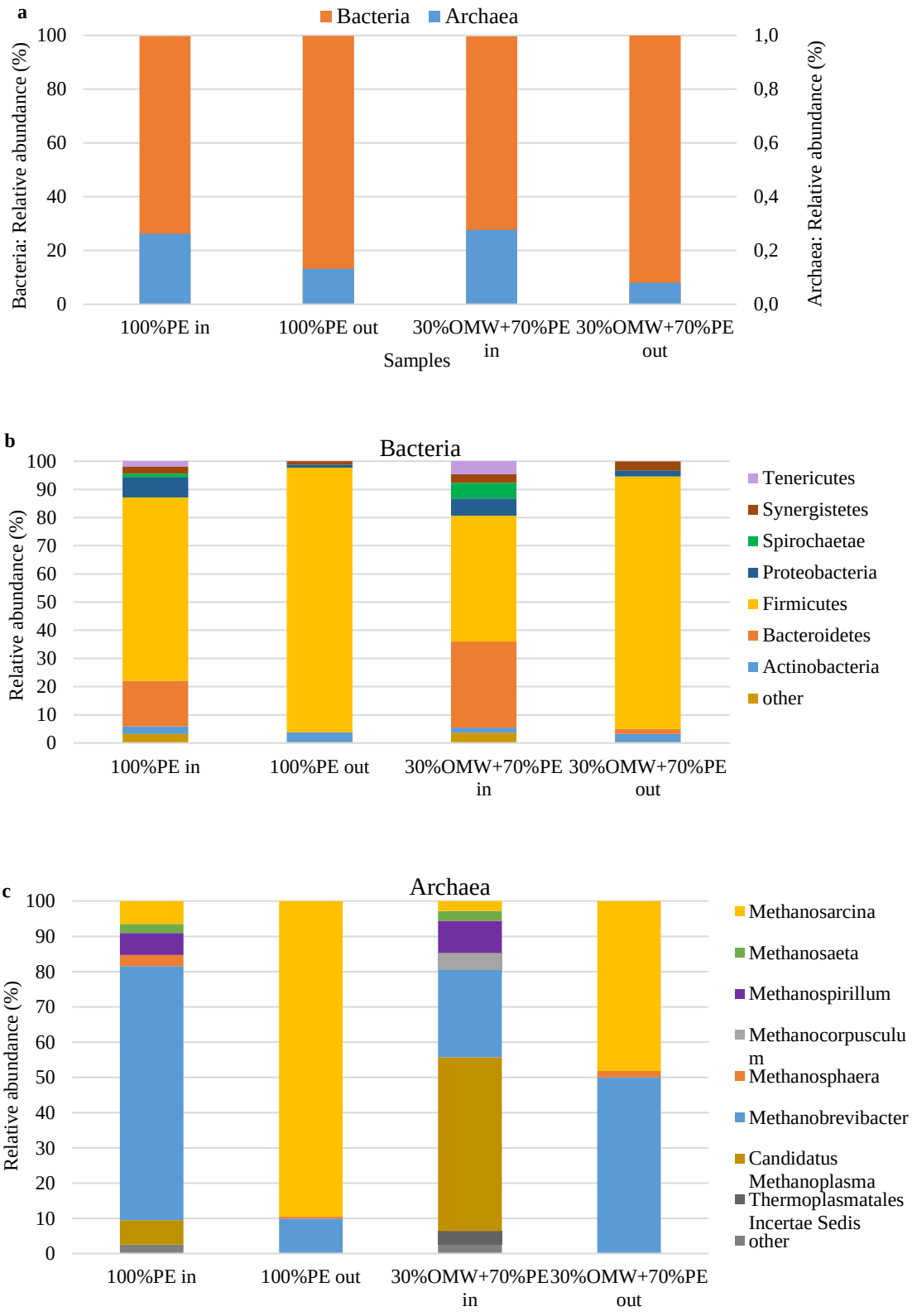

Fig. 3.7.: Relative abundance of Bacteria and Archaea domains (a), and their respective phyla (b) and genera (c), from the first experiment

3.5.2.3. Second essay

The 70%BWW+I (in and out) mixture was selected to study the molecular biodiversity because it produced the highest amount of biogas. As expected, the Bacteria were more abundant than Archaea, although it was observed a minor population increase in the latter domain (from 0.3% to 0.7%; see Figure 3.8.a).

Proteobacteria was the dominant phylum of Bacteria domain throughout the experiment, with Firmicutes and Chloroflexi as sub-dominant (Figure 3.8.b). All the phyla had minor changes in their relative abundance, but maintained relatively stable. However, in Proteobacteria, Rhizobiales replaced Pseudomonadales as the most abundant order (Appendices figures 6.d. and f.). According to Nelson *et al.*, 2011, Proteobacteria is responsible for oxidization of propionate to acetate used by acetoclastic methanogens, and Firmicutes degrading more complex molecules (mentioned earlier).

Concerning the reddish pigmentation, the presence of PNSB was searched, through sequence homology analysis. The presence of the bacterial genus *Rhodobacter* (identified with 97% similarity) was found in inoculum, with a relative abundance of 0.73% in relation to all detected populations (Figure 3.9.), which maintained after the anaerobic process. The same process, which occurred under light intensity, together with high concentration of acetate in the beginning of anaerobic digestion (1.3 g L^{-1}) might explain the growth of Rhodobacter populations and the red pigments production.

The *Methanosaeta* genus represented almost all the Archea population in 70%BWW+I mixture (see Figure 3.8.c.), being *Methanolinea* the only genus represented aside from the former. The presence of *Methanosaeta* in the mixture suggests that, according with Cho *et al.*, 2013, Wang *et al.*, 2017 and Wang *et al.*, 2018, the acetoclastic pathway (i.e. producing methane from acetate) was the main way for methane being formed during the experiment.

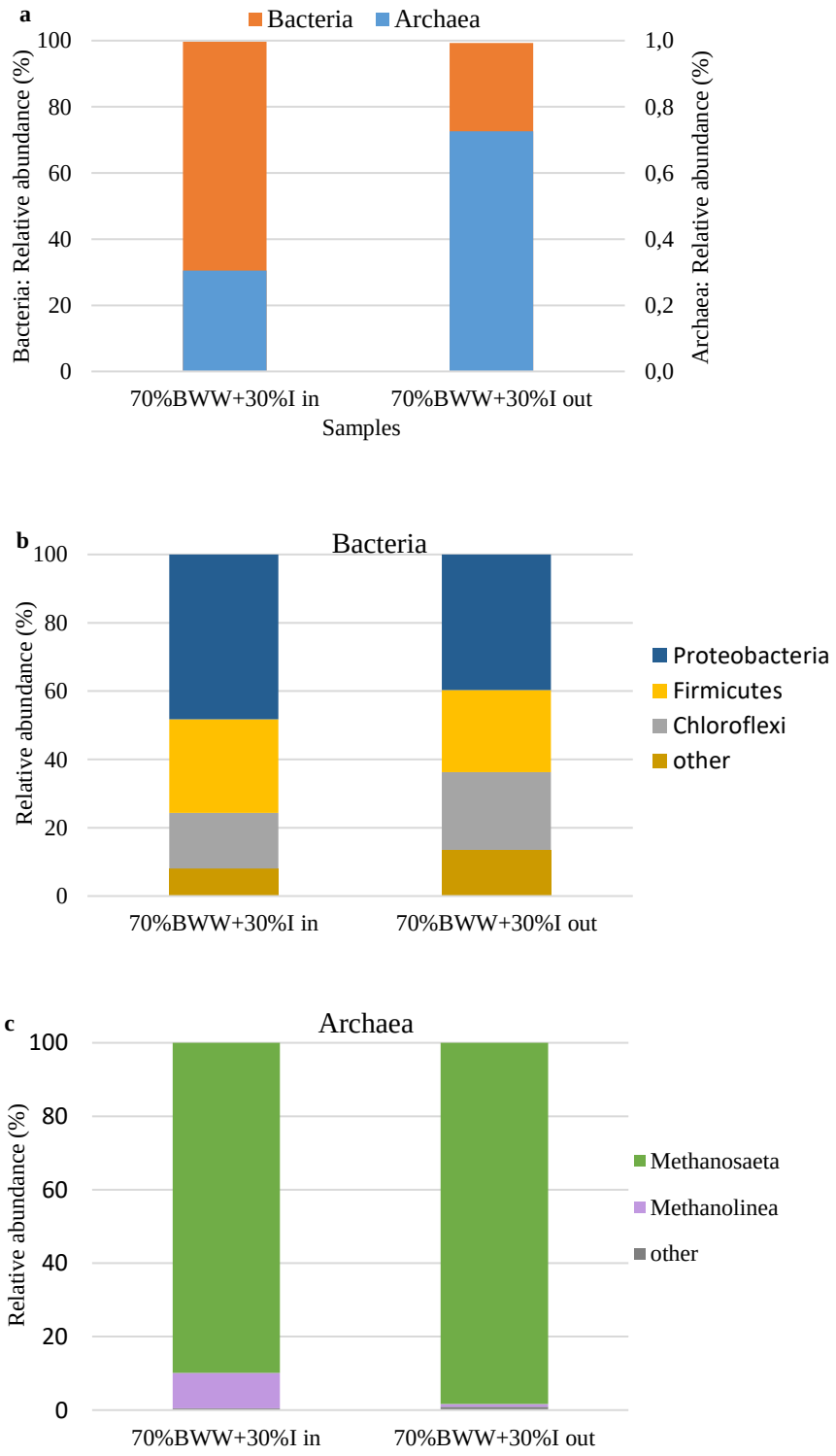

Fig. 3.8.: Relative abundance of Bacteria and Archaea domains (a), and their respective phyla (b) and genera (c), from the second experiment

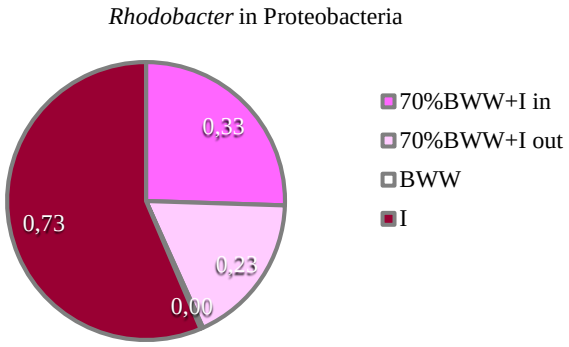

Fig. 3.9.: Relative abundance of *Rhodobacter* populations in the effluents and in experiment mixtures

3.5.2.4. Third essay

The majority of microorganisms presented in the substrate belonged to Bacteria domain, with 0.7% represented by Archaea (Figure 3.10.a.). During anaerobic digestion the Archaea population decreased during the second phase (HRT = 3.0), and later recovered to previous numbers (in the last phase, when HRT = 1.0).

The populations in the substrate were diverse on Bacteria domain (Figure 3.10.b.), but most of them belonged to phylum Firmicutes (40.9%), mostly represented by Clostridiales, (35%, Appendices figure 6.c.), which became predominant throughout the experiment (up to 55% on the last phase), with Bacteroidetes and Proteobacteria as sub-dominant (the latter only at second phase). Moreover, the population was slightly more diverse than the other essays. However, it was possible to observe the Bacteroidetes had similar representation on the substrate prior to digestion, but it decreased afterwards, further implying that Firmicutes and Bacteroidetes may have competed over the same resources on the reactor, with the former prevailed over the latter, such as happened in the first essay. The phyla that were detected between 1% and 10% weren't considered in this discussion.

As what was observed in Bacteria domain, the substrate also had a variety of Archaea genera, but only the *Candidatus Methanoplasma* and *Methanobrevibacter* were the predominant (39.9% and 29.5% in relative abundance, respectively, Figure 3.10.c.). After the anaerobic digestion of the substrate, big shifts on microbial population had occurred, with *Methanosaeta* being the dominant genus, with at least 83% of archaea population belonged to the genus in both collected samples. This also implies that the acetoclastic metabolism was the main pathway the methane was formed during the experiment (Cho *et al.*, 2013; Wang *et al.*, 2017 and Wang *et al.*, 2018). Other genera weren't considered due to their low presence detected presence (below 10% in relative abundance).

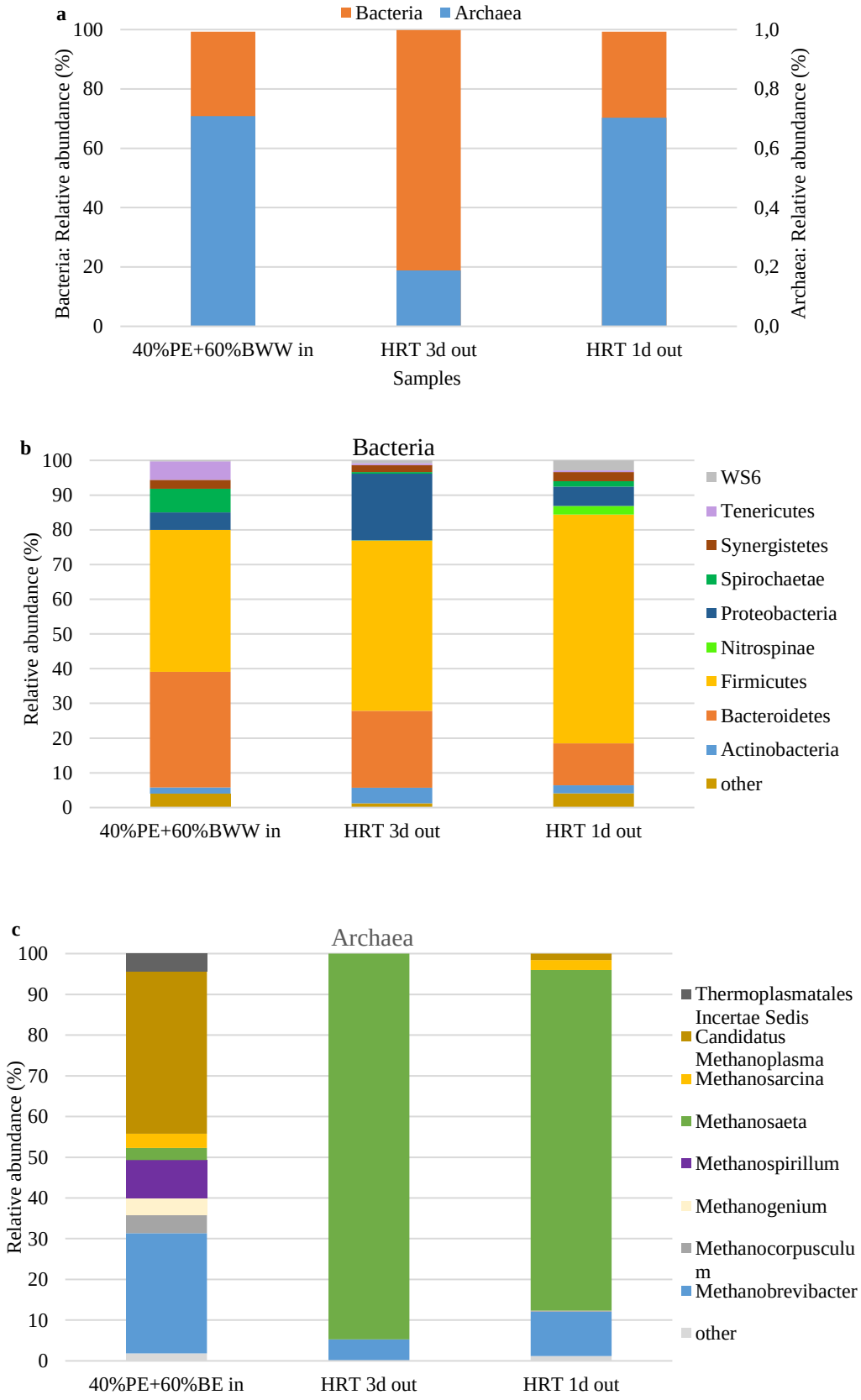

Fig. 3.10.: Relative abundance of Bacteria and Archaea domains (a), and their respective phyla (b) and genera (c), from the third experiment

4. CONCLUSIONS

From the recorded data of batch experiments, under tested experimental conditions, it is possible to infer that OMW had a high inhibitory capacity over anaerobic digestion microorganisms. This was more notorious when OMW was digested with a diluted effluent (BWW, second essay), during which the biogas/methane productions were below the value of 70 mL. When PE was used to complement OMW, the high organic content of PE allowed to counteract the toxic compounds of OMW and make the stable conditions for the microbial consortium to degrade the effluents and produce biogas. However, this was possible at lowest tested concentrations of OMW (30% v/v) in the mixture, in which a production of about 780 mL was obtained. Applying a OMW proportion of 50% (v/v), a period of approximately 30 days was required to record increases in gas production that did not exceed the mean value of 330 mL. From the recorded data it is possible to infer that the tested PE is a good complementary substrate to treat OMW through anaerobic digestion.

Concerning the hybrid anaerobic reactor (detailed on subchapter 3.4), working under semi-continuous mode, it was able to withstand high amounts of substrate influx, with high organic loading rates (5.2, 10.0 and 33.6 kg m^{-3} d^{-1}), without suffering any negative effects, such as biomass washout or loss of the microbial population stability, being able to remove organic compounds from substrate and produce biogas and methane (1.2, 2.3 and 3.0 L $L^{-1}d^{-1}$ with 63.8, 79.5 and 79.5% CH_4, respectively). So, the tested PE can be mixed with BWW and both be treated through anaerobic digestion.

From the molecular analysis, an evolution of microbial population was observed during the experiments towards a specialization to the operational conditions in the anaerobic processes, involving the loss of the initial population diversity.

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APPENDICES

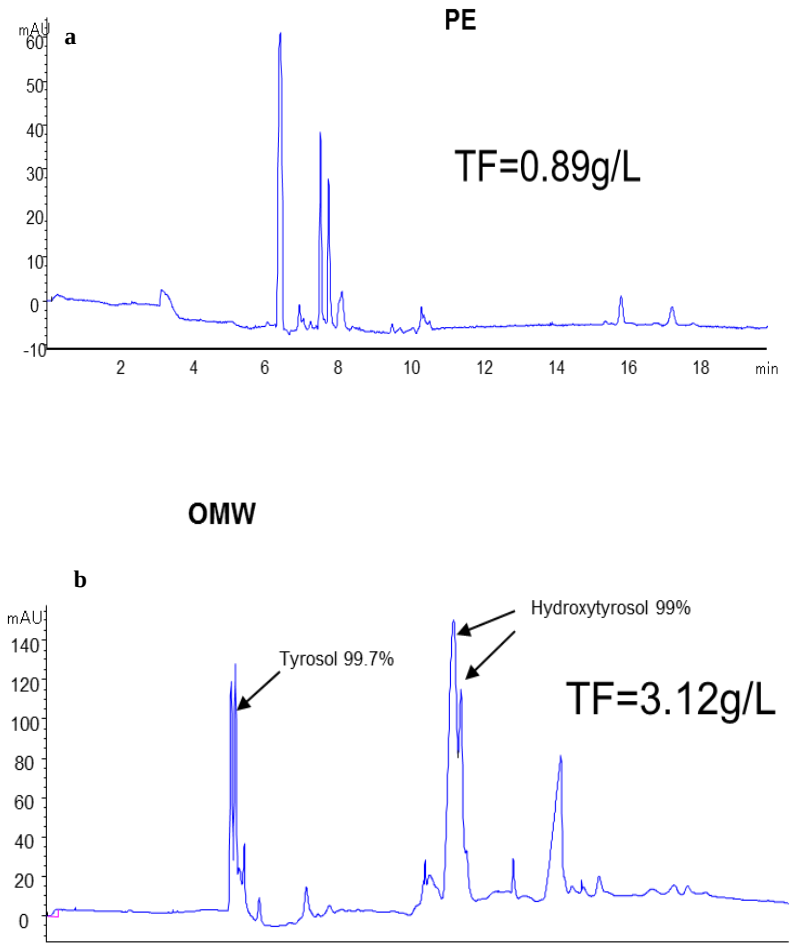
Submitted works:

- A. Neves, L.B. Roseiro, L. Ramalho, A. Eusébio, I.P. Marques. 2019. Hybrid anaerobic reactor: brewery wastewater and piggery effluent valorisation. Full paper. Proceedings of the 5thInternational Conference WASTES: Solutions, Treatments and Opportunities, Lisbon, September 4–6 (Oral presentation).

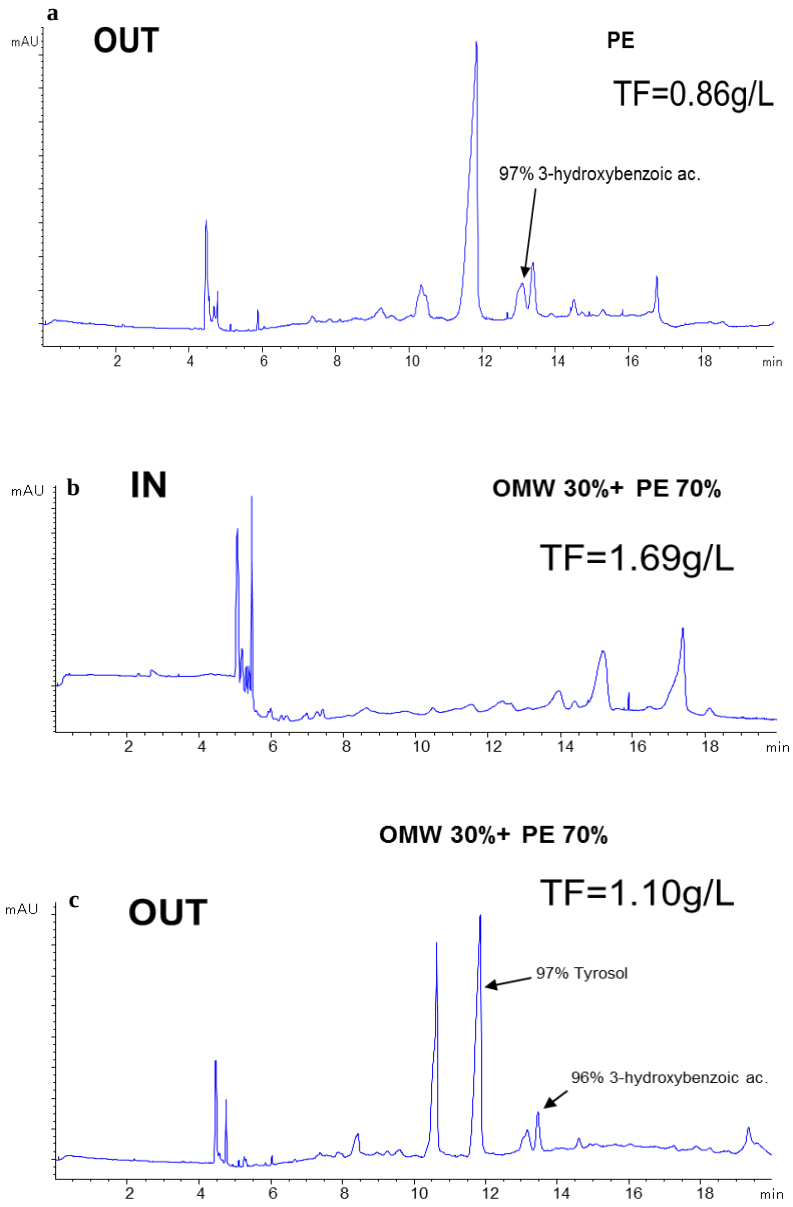
- A. Neves, L. Ramalho, L.B. Roseiro, A. Eusébio, I.P. Marques. 2019. Biogas: olive mill wastewater as complementary substrate of piggery effluent. Full paper. Proceedings of the 5thInternational Conference WASTES: Solutions, Treatments and Opportunities, Lisbon, September 4–6 (Poster).

- A. Neves, L. Ramalho, I.P. Marques, A. Eusébio. 2019. *Rhobacter* and pigments in anaerobic digestion of brewery effluent. Full paper. Proceedings of the 5thInternational Conference WASTES: Solutions, Treatments and Opportunities, Lisbon, September 4–6 (Poster).

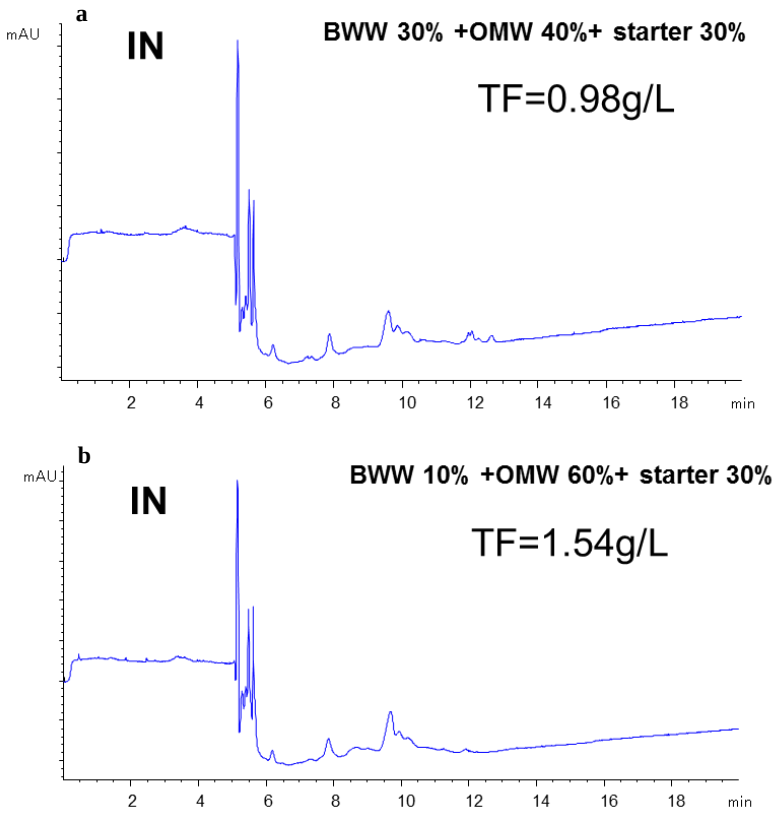
Phenolic content: electropherograms



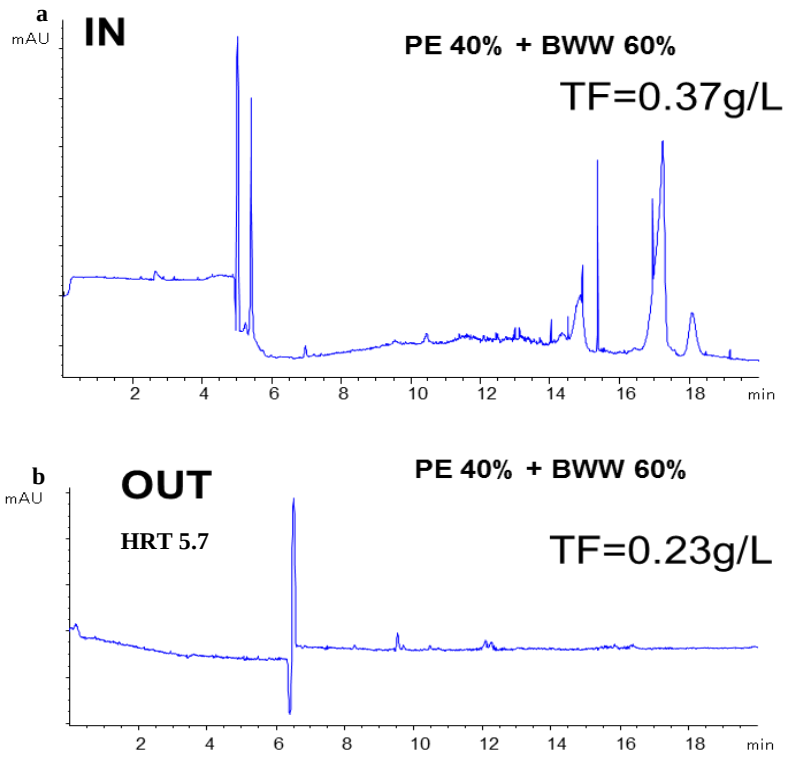
Appendices figures 1.a. and b.: Electropherograms of the effluents



Appendices figures 2.a. to c.: Electropherograms of the first essay

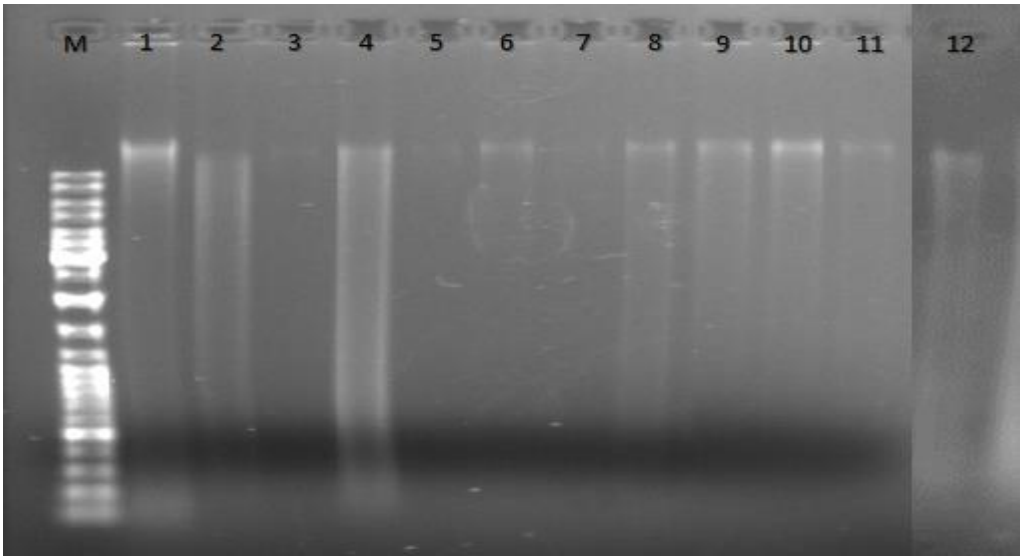


Appendices figures 3.a. and b.: Electropherograms of the second essay

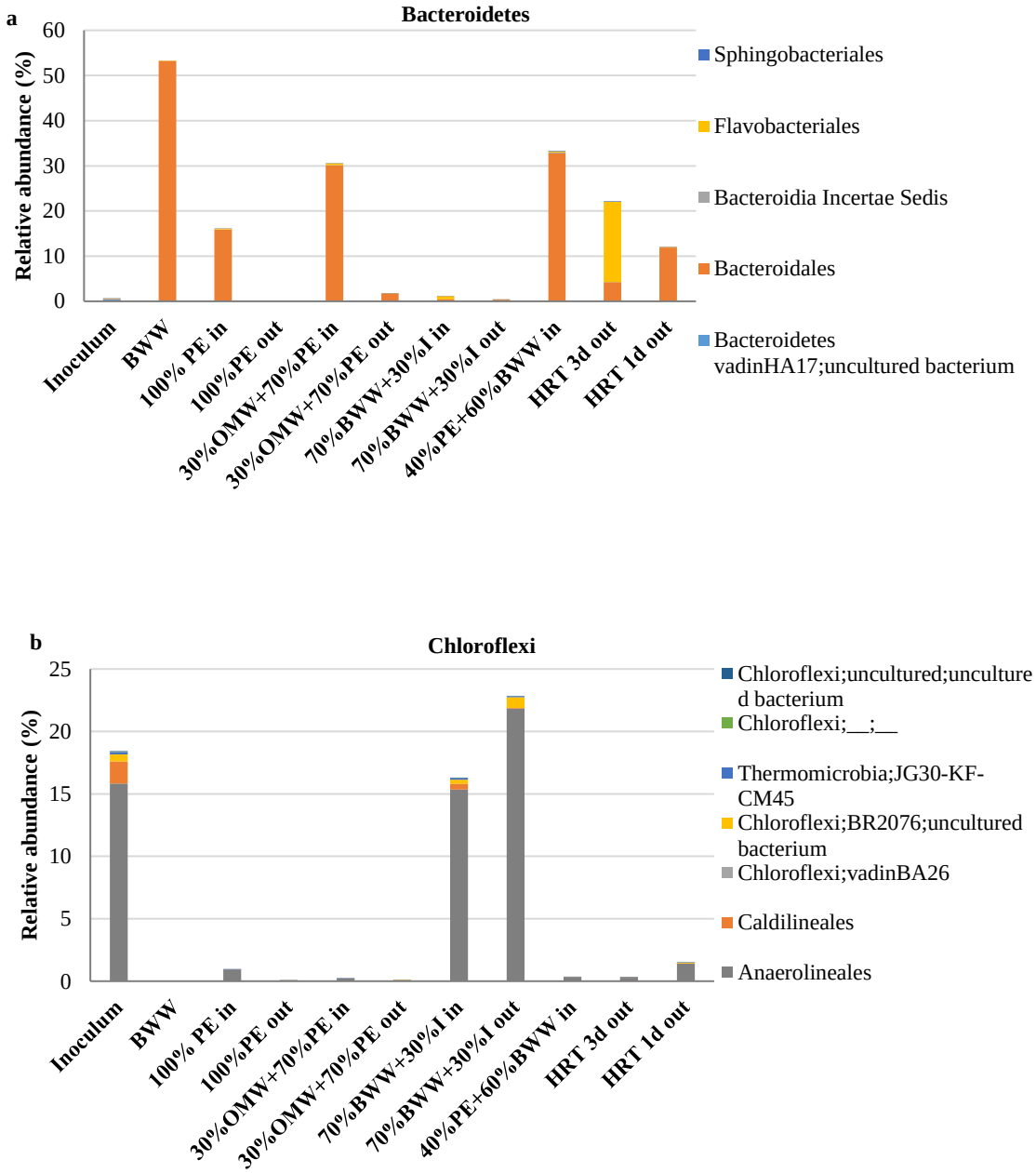


Appendices figures 4.a. and b.: Electropherograms of the third essay

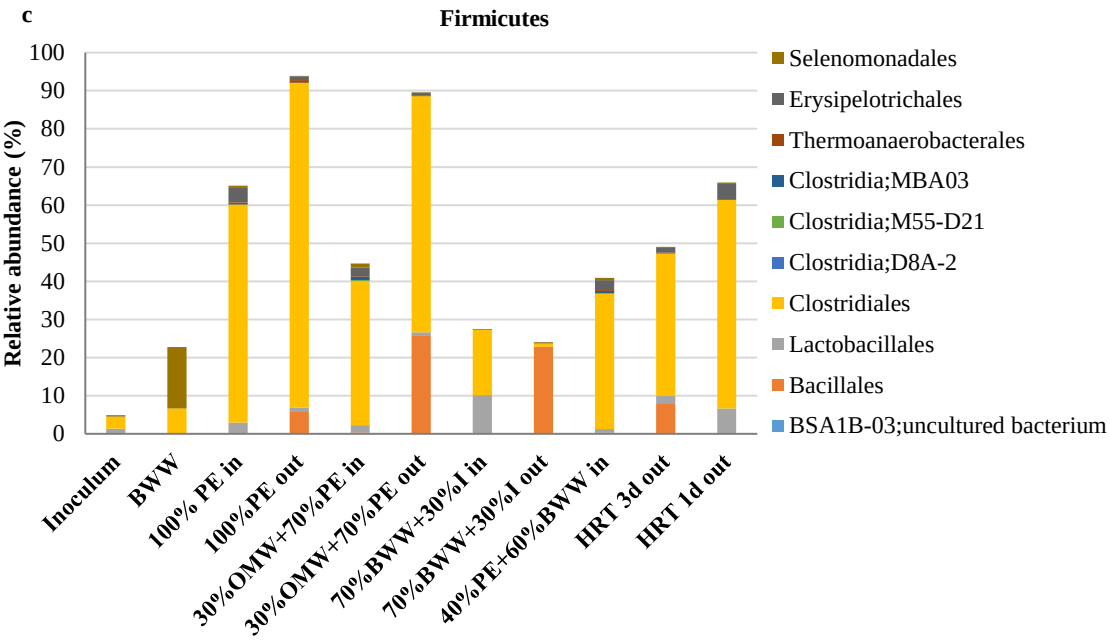
DNA extraction and molecular analysis

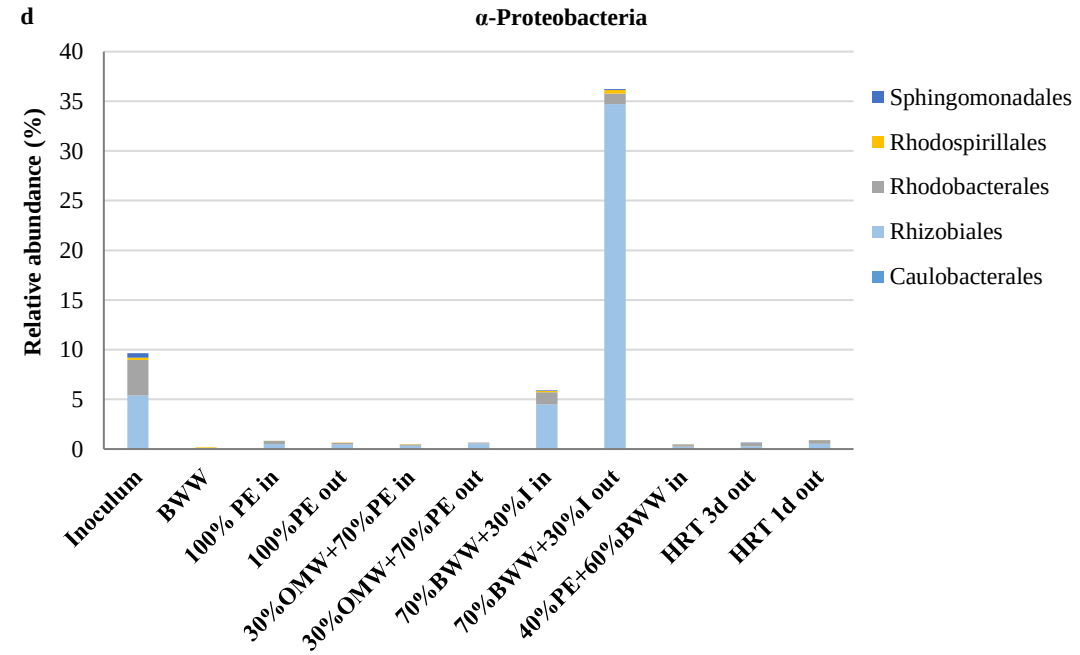


Appendices figure 5: Electrophoresis gel (1.0% agarose) stained with 3% GreenSafe Premium (40 min, 100 V). M: DNA Ladder V (NZYTech, Portugal); 1-Inoculum; 2-BWW; 3-100%PE out; 4-30%OMW+70%PE in; 5-30%OMW+70%PE out; 6-70%BWW+30%I in; 7-70%BWW+30%I out; 8-40%PE+60%BWW; 9-HRT 5.7d out; 10-HRT 3d out; 11- HRT 1d out; 12-PE

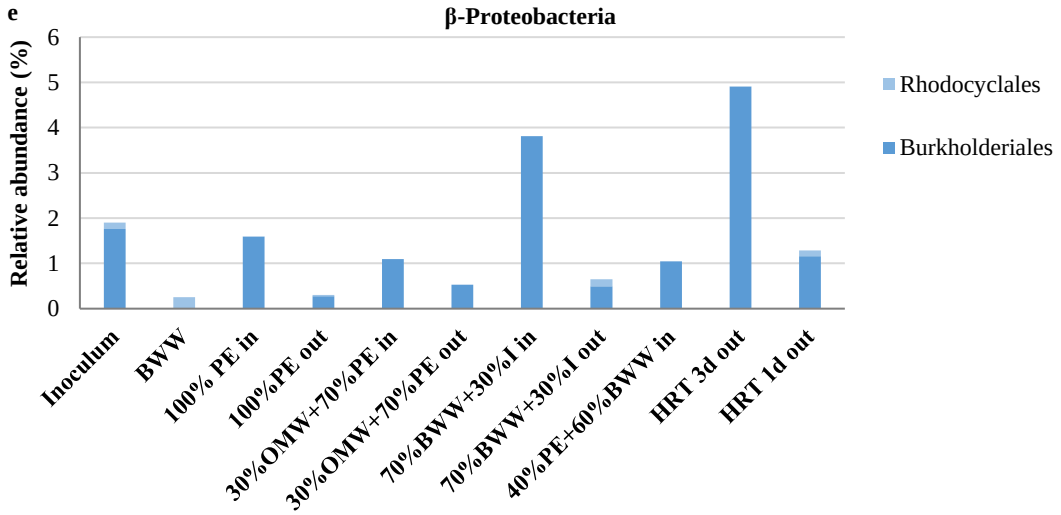


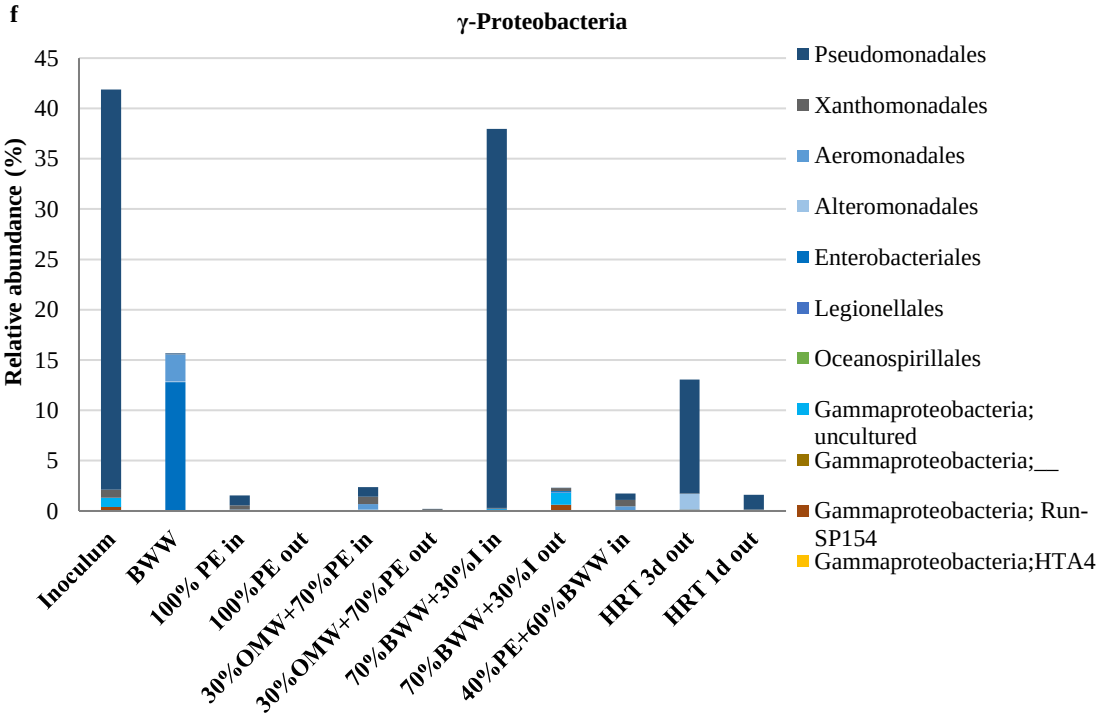
Appendices figure 6.a. and b.: Details about relative abundances of (a) Bacteroidetes and (b) Chloroflexi composition by order in samples from anaerobic digestion experiments. The abundance is presented in terms of a percentage of the total number of sequences in a sample



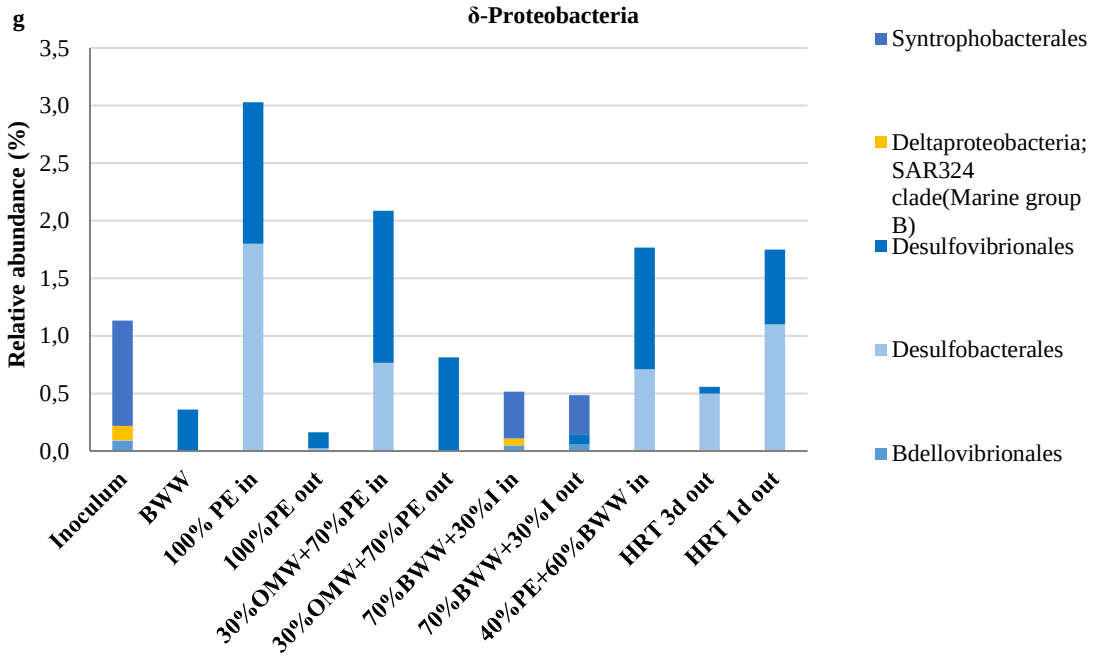


Appendices figure 6.c. and d.: Details about relative abundances of (c) Firmicutes and (d) α-Proteobacteria composition by order in samples from anaerobic digestion experiments. The abundance is presented in terms of a percentage of the total number of sequences in a sample





Appendices figure 6.e. and f.: Details about relative abundances of (e) β-Proteobacteria and (f) γ-Proteobacteria composition by order in samples from anaerobic digestion experiments. The abundance is presented in terms of a percentage of the total number of sequences in a sample



Appendices figure 6.g.: Details about relative abundances of δ-Proteobacteria composition by order in samples from anaerobic digestion experiments. The abundance is presented in terms of a percentage of the total number of sequences in a sample